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COMBATING MALARIA

a three-step approach towards elimination

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COMBATING MALARIA

a three-step approach towards elimination

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CHAPTER 1

GENERAL INTRODUCTION

Parts of this chapter have been published in:
Stappen op weg naar de uitroeiing van malaria / On the way to eradicating malaria
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Malaria

Malaria is a life-threatening disease caused by the protozoan *Plasmodium* parasite. It accounts for 207 million clinical cases a year worldwide with an estimated 627,000 deaths, with children under five years of age in sub-Saharan Africa most severely affected [1]. Severe anaemia, cerebral malaria, acute respiratory distress syndrome and organ failure are the most common causes of death [2-4]. The malaria parasite *Plasmodium falciparum* that predominates in sub-Saharan Africa is responsible for the vast majority of these deaths [1]. Besides *P. falciparum*, four other *Plasmodium* parasites (*P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*) can cause malaria in humans after bites by infected female mosquitoes of more than 30 anopheline species [1,5]. The *Plasmodium* life cycle is given in Figure 1 and shows two distinct parasite phases, an asexual phase where parasites are replicating in the human host and a sexual phase where fertilisation of sexual stage parasites takes place inside the mosquito midgut.

The highest malaria prevalence and transmission intensity is currently found in the World Health Organization's African region where approximately 80% of cases and 90% of deaths are estimated to occur [1]. Although here prevalence [6-8] as well as malaria mortality is declining [1,9], it remains a huge problem with tremendous economical consequences [10,11] particularly affecting the poorest populations and rural areas [1,12].

Malaria control and eradication: the past and present

In 1955 the World Health Organization (WHO) adopted a Global Malaria Eradication Programme based on the widespread use of the insecticide DDT (dichlorodiphenyltrichloroethane) against *Anopheles* mosquitoes and the antimalarial drug chloroquine for treatment and elimination in humans [13]. Although these efforts were successful over large geographical areas including the Netherlands, the campaign was only launched in three countries of sub-Saharan Africa [13]. It was not considered feasible in the others particularly due to the very high infection pressure and poor (health) infrastructure and underperforming health services. Furthermore, the worldwide heterogeneity of malaria transmission and disease was underestimated [13-15]. In 1969 global malaria eradication was concluded to be unfeasible: for areas where malaria continued to be endemic, the focus shifted to control of (severe) malaria disease [16]. This immediately resulted in a considerable reduction in financial support to antimalarial programmes, which was aggravated by economic and financial crises in the 1970s and 1980s [13,14].

In the 1980s and 1990s malaria started a gradual or sometimes dramatic resurgence, which was primarily fuelled by the increase and spread of chloroquine-resistant parasites and DDT-resistant anophelines [15,17]. There was at least a doubling of childhood malaria-specific mortality from the 1980s compared with the early 1990s in various areas in sub-Saharan Africa [17,18] and a rise in the proportion of all childhood deaths attributed to

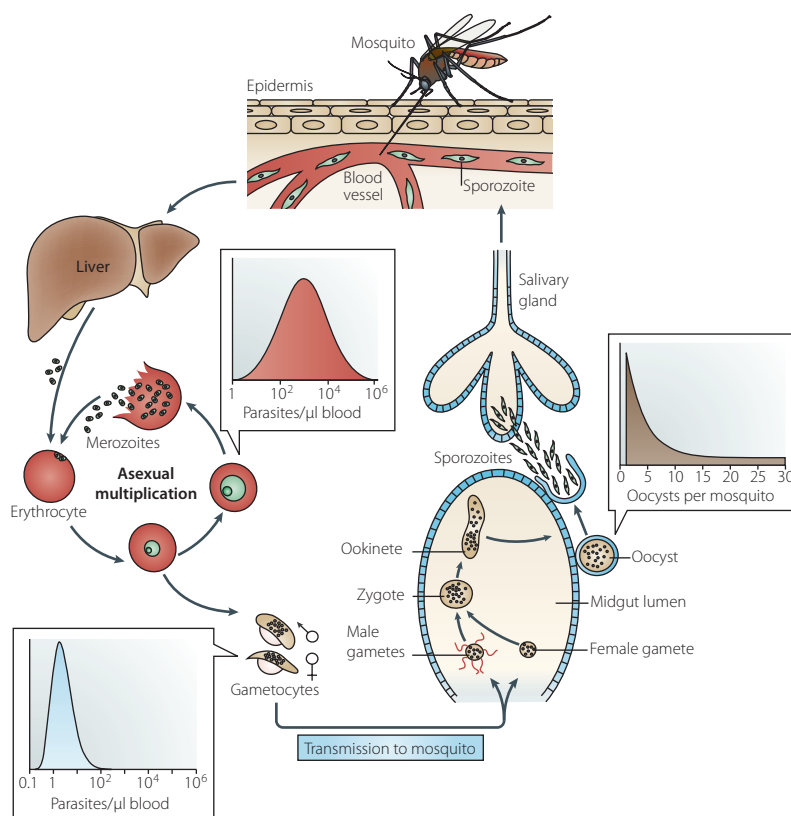


Figure 1 Life cycle of *Plasmodium* parasites

Female *Anopheles* mosquitoes inject invasive forms of malaria parasites - sporozoites - when taking a blood meal from the human host. These sporozoites migrate via the skin and blood vessels to the liver where they mature in hepatocytes without giving clinical symptoms. After ~1 week, an estimated 40,000 merozoites per infected hepatocyte are released in to the circulation and rapidly invade erythrocytes, where they again mature and eventually rupture the erythrocyte while releasing 16–32 new merozoites into the circulation. These new merozoites again invade erythrocytes, leading to an exponential cyclical increase in parasitaemia with clinical symptoms. Each cycle takes ~48 hours to complete. The variation in the density of asexual parasites in the bloodstream of infected individuals in an area of moderate transmission intensity is shown in red (inset).

A fraction of asexual parasites transforms into sexual stage parasites, gametocytes. The density of mature gametocytes in peripheral blood is typically less than 100 gametocytes per μ l of blood (light blue; inset). Once infectious gametocytes have been ingested by a blood feeding female *Anopheles* mosquito, male and female gametes fuse to form an motile ookinete in the mosquito midgut that can penetrate the mosquito midgut wall to form oocysts. The oocyst eventually releases new sporozoites that migrate to the mosquito salivary gland, ready to infect a new human. Oocyst densities in naturally infected mosquitoes that have fed on either microscopic or submicroscopic parasite carriers are typically in the range of 1–10 oocysts (brown; inset). Sporogonic development renders mosquitoes infectious to humans after approximately 11–16 days [Meis JF, et al. 1992].

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malaria from 18% pre-1960 to 30% during the 1990s [18]. At the end of the 1990s, the malaria situation in several settings may have been further deteriorated as a result of civil disturbances, increased travel and the HIV epidemic [13,19].

At the beginning of the new millennium, greater recognition of the devastating socio-economic effects of malaria stimulated investments in malaria control programmes and malaria research. From then on, international disbursements to malaria-endemic countries have increased tenfold to US\$ 1.71 billion in 2010 and US\$ 2.5 billion in 2012 [1]. This is an important step forward but still less than half of the estimated US\$ 5.1 billion required annually to achieve universal access to malaria interventions [1].

The tremendous expansion in the financing and implementation of malaria control programmes in the past decade resulted in reductions in malaria case incidence, morbidity and mortality [1,8,9]. The number of clinical malaria cases dropped from 226 million in 2000 to 207 million in 2012 and the number of deaths fell from 985,000 in 2000 to 627,000 in 2012 [1]. Malaria transmission intensity decreased in several endemic areas and many former high-endemic malaria areas are currently characterized by low or decreasing malaria transmission [1,8,20-22].

Despite the progress much greater gains could be achieved against malaria, including elimination from a number of countries and regions by aggressively scaling up control with currently available tools and strategies. Nonetheless, even with maximal effort elimination in many areas and global eradication is considered unfeasible [23,24] partly due to constant threats to current progress, such as emergence of artemisinin resistance [25-30] and vector mosquito resistance to insecticides [31]. Consequently, need for more potent tools to contain dissemination of resistant parasite strains and accelerated development of vaccines that can impact on malaria incidence, disease, and death remains a high priority [32,33].

Improving access and accuracy of diagnosis using malaria rapid diagnostic tests

One of the main threats to current progress in decreasing the malaria burden is over-treatment with antimalarials, which creates artemisinin drug pressure [28,34] that may facilitate the spread of artemisinin resistant parasites. Since the 1980s, malaria control has incorporated presumptive treatment of fever with antimalarials, treating all febrile episodes suspected of malaria with a full therapeutic dose of antimalarials without being confirmed by laboratory diagnosis [19]. This approach is no longer justifiable now it is realized that treatment decisions are based on clinical signs and symptoms that are neither sensitive nor specific [35]; the initial symptoms of malaria may include tachycardia, tachypnoea, chills, malaise, fatigue, diaphoresis, headache, cough, anorexia, nausea, vomiting, abdominal pain, diarrhoea, arthralgias, and myalgias [36]. The issue of most concern for patients with non-malaria severe febrile illness is that treatable alternative

diagnoses are being missed, and in febrile children it was shown that mortality due to bacterial disease is underestimated [37,38].

Reports from the past decade indicate that many parts of Africa are currently characterized by low or decreasing malaria transmission intensity [6-9,20,39]. Particularly in these low and moderate transmission areas presumptive treatment practices result in extensive overuse of antimalarial drugs, which can contribute to a reduced susceptibility of parasites to artemisinins [27,28], the core compound in the world's most effective antimalarial medicines [1,27]. Improving access to and accuracy of diagnosis may thereby prove crucial to prevent overtreatment and stimulate the search for alternative causes of illness.

The gold standard for malaria diagnosis, microscopy, faces challenges particularly in rural clinics: microscopes and reagents are often of poor quality and there is a lack of well trained microscopists [20,22,40,41]. Malaria rapid diagnostic tests (RDTs) - immunochromatographic tests that detect malaria parasite antigens in whole blood samples - pose an attractive alternative to routine microscopy: they are sensitive and specific by detecting specific *P. falciparum* or other *Plasmodium* antigens [42-45], are simple in comparison with other malaria diagnostic tests because they do not require electricity or expensive equipment, provide results in 15–20 minutes, are relatively inexpensive at \$0.30–1.00 per test [46], are easy to use, and provide more objective results than microscopy - the test line is either present or absent. RDTs are one of the fundamental pillars of the existing global strategy to fight malaria [47] and have the potential to reduce the burden of malaria by improving parasite-based diagnosis. Improved diagnosis, in turn, can improve treatment outcomes, rationalize health care costs by reducing drug consumption [48] and minimize drug pressure [28,34], and assist in monitoring disease trends [47]. In theory, the focus on RDTs should change presumptive treatment based on signs and symptoms alone, to parasite-based diagnosis and treatment based on test results. It is, however, unclear whether a change in policy directly affects routine practice or if additional measures are needed to improve implementation.

Improving vector control by mosquitocidal drugs

Insecticide-treated bed nets (ITNs) and indoor residual spraying (IRS) were two of the conventional key interventions that were rapidly scaled-up in the past decade [49-52] and that contributed to the decline in *P. falciparum* malaria in a number of endemic settings throughout sub-Saharan Africa [1,6-9]. Here, the proportion of the population with access to an ITN in their household increased dramatically from 2005 to 2011 although the rate flattened during the last 2 years, reaching 42% in 2013 [1]. ITNs have been shown to reduce malaria transmission by 70–90%, leading to significant reductions in human-vector contact, the number of infectious mosquito bites [53,54] and the force of infection [55]. IRS targets the vector, the *Anopheles* mosquito. Despite negative effects on the environment,

dichlorodiphenyltrichloroethane (DDT) has been proven to be very successful for disease vector control [50,51] and there will be a continued role for DDT in malaria control despite its toxicity [56,57].

The efficacy of both interventions is being threatened by *Anopheles* mosquito resistance to insecticides [31]. Furthermore, there are data indicating that mosquitoes can increase their biting behaviour from indoors to outdoors [58]. It is therefore debated whether up-scaling of conventional malaria control measures like ITNs and IRS will sustain declines in malaria or achieve malaria elimination [59] unless augmented by tools that specifically aim to reduce transmission.

The transmission of malaria from humans to mosquitoes depends on the presence of mature gametocytes in the peripheral blood [60]. Gametocytes are relatively insensitive to schizonticidal drugs and current first line antimalarial drugs may therefore need to be supported by specific transmission-blocking drugs [61]. Traditionally, the search for transmission-blocking drugs has focused on antimalarial drugs with gametocytocidal activities. Primaquine has received considerable interest in this because it is the only currently available drug that actively clears mature gametocytes, which can persist for several days after infected individuals are treated with artemisinin-combination therapy (ACT) [62-65]. The average duration of gametocyte carriage after ACT is estimated at 13.4 days although gametocyte densities are highest in the first week after initiation of treatment [66]. As a result, a substantial proportion of patients can transmit malaria up to 7-14 days after successful ACT treatment [65,67-69]. Although addition of primaquine can prevent malaria transmission from infected humans to mosquitoes after treatment [70], it is causally associated with intravascular haemolysis in glucose-6-phosphate-dehydrogenase (G6PD) deficient individuals [71-76], presenting a barrier for primaquine acceptance in combination with ACTs [28,77].

An alternative line of thinking to prevent post-treatment malaria transmission was recently suggested by several reports on insecticidal capacity of ivermectin (IVM), a drug with broad-spectrum activity against nematodes and ectoparasites [78]. It has been shown to reduce the lifespan of malaria-transmitting *Anopheles* mosquitoes that feed on humans who have taken IVM [79-81]. In neuronal and neuromuscular tissues of invertebrates IVM activates glutamate-gated chloride (GluCl) channels, causing flaccid muscle paralysis that can lead to death of the insect [82,83]. GluCl channels are not present in mammals [82] therefore IVM has an excellent safety profile and it has been used for decades in mass treatment campaigns to reduce the burden of onchocerciasis [84]. This makes IVM a potentially attractive component of malaria control efforts, where it could be part of a drug combination that not only treats malaria patients but also reduces onward transmission of the disease. The duration and the impact of the mosquitocidal effect of IVM is key to its potential role as an adjunct malaria therapy. However, few data are available on the exact duration and the impact of this effect.

Protection against malaria by vaccination

Vaccines represent one of the most important global health investments and are the most cost-effective tools for public health. According to the World Health Organization, immunizations save an estimated 2.5 million lives every year from diphtheria, tetanus pertussis and measles [85-88]. An effective vaccine against malaria has long been envisaged as a valuable addition to the available tools for malaria control and compulsory to achieve malaria eradication. However, the complexity of the malaria parasite makes development of a malaria vaccine a very difficult task. Given this, there is currently no licensed malaria vaccine, despite decades of intense research and development effort [1].

The agenda for malaria vaccine development is determined by the different stages of the parasite's lifecycle (Figure 1). Due to differences in morphology, composition, physiology and patho-physiology of each of the parasite's stages, stage-specific vaccines will have different effects: clinical illness will be prevented by pre-erythrocytic vaccines targeting liver-stage parasites [89] or a vaccine that targets the asexual blood-stage of the parasite [90,91], while transmission-blocking vaccines will specifically target malaria sexual stage antigens, reducing transmission from human to mosquito [63,92].

Vaccine development is hampered by genetic and antigenic diversity of the *Plasmodium* parasite and the complex interaction between parasite and human immune system [93]. Around 20 candidate vaccines are currently being evaluated in Phase 1 or Phase 2 clinical trials. All are based on one or a limited number of *Plasmodium* proteins. Only one candidate vaccine is currently tested in Phase 3 clinical trials: the RTS,S/AS01-vaccine which comprises a fusion protein of the sporozoite circumsporozoite antigen with hepatitis B surface antigen, and includes the potent AS0 adjuvant. The vaccine showed good safety in African children aged 6–12 weeks but only moderate efficacy, with 30% protection against clinical malaria and 26% protection against severe malaria in the 12 months after the last dose [94]. Previously reported results in slightly older children (aged 5–17 months) were better, with 55% protection against all falciparum malaria and 35% protection against severe malaria during 14 months [95]. Although protection is incomplete, RTS,S could be considered a breakthrough in malaria vaccine development.

A different approach in malaria vaccine development is based on the whole *P. falciparum* sporozoite. In the 1970s it was shown that immunization of volunteers by bites of mosquitoes infected with irradiated *P. falciparum* sporozoites induces sterile and sustained protection [96-100]. More recently, it was shown that exposure of healthy malaria-naïve volunteers while taking chloroquine prophylaxis to *P. falciparum* sporozoites via three times 12–15 infected mosquito bites (ChemoProphylaxis and Sporozoites (CPS) immunization) induced sterile protection against a homologous challenge infection [101]. This protection was found to be long-lasting: after 2.5 years 4/6 volunteers were still protected [102]. It is unclear whether immunity induced by CPS targets pre-erythrocytic

P. falciparum stages, blood-stages or both. Furthermore, this highly protective CPS immunization strategy is currently unsuitable as an implementable vaccine in the field, because it depends on inoculation of sporozoites by mosquito bites. Replacing mosquito bites by needle and syringe injection is a crucial step in translating CPS immunization into an implementable whole sporozoite vaccine approach. For this, considerable technological challenges regarding production, formulation and delivery of such a vaccine will have to be overcome, as well as the safety aspect that is a considerable issue.

Aims and outline of the thesis

The main objective of this thesis is to evaluate currently available and experimental treatment and vaccination strategies that have the potential to support malaria control, elimination, and eradication efforts now and in the future. The work described in this thesis can be grouped into three main sections. In the first section the implementation of malaria rapid diagnostic tests, a current strategy to support diagnosis and accurate treatment, is assessed. In the second and third section near future and future strategies are tested, respectively, the mosquitocidal effect of ivermectin and the potential of whole sporozoite vaccination.

Improving access and accuracy of diagnosis using malaria rapid diagnostic tests

Presumptive treatment of suspected malaria cases may no longer be a justifiable approach in an era of decreasing malaria transmission and reduced malaria mortality. Malaria rapid diagnostic tests (RDTs) have the potential to play an important role in rationalising malaria treatment practices but their impact depends on a multitude of aspects including their availability and diagnostic practices. In theory, the availability of these tests may allow for a switch from presumptive treatment to parasite based diagnosis and treatment based on the result of the test. Specific aims regarding implementation of RDTs were:

- to assess the impact of a government policy change, comprising the provision of malaria RDTs, on clinical decision-making in district hospitals in Tanzania (Chapter 2)
- to identify technical and operational challenges to achieving at-scale implementation of malaria RDTs in sub-Saharan African settings (Chapter 3)

Mosquitocidal additions to current antimalarial treatment to prevent transmission

Ivermectin (IVM) is a drug with broad-spectrum activity against nematodes and ectoparasites and is widely used in mass treatment campaigns against onchocerciasis. Evidence in literature suggests that IVM can reduce the lifespan of malaria-transmitting *Anopheles* mosquitoes, hence onward malaria transmission, after feeding on a host who has taken this drug. We addressed the following objectives:

- to acquire a precise estimate of the duration of the mosquitocidal effect of IVM (Chapter 4)
- to determine the safety and efficacy of IVM in combination with conventional malaria therapy to prevent malaria transmission from *Plasmodium falciparum* infected humans (Chapter 5)

Protection against malaria by inoculation of whole sporozoites

Malaria naive volunteers immunized under chloroquine ChemoProphylaxis with *Plasmodium falciparum* Sporozoites (CPS) have shown to develop complete, long-lasting protection against subsequent challenge by bites of *P. falciparum*-infected mosquitoes. It is unclear whether immunity induced by CPS targets pre-erythrocytic *P. falciparum* stages, blood-stages or both. Furthermore, mosquito bites will have to be replaced by needle and syringe injection in order to translate CPS immunization into an implementable whole sporozoite vaccine approach. We explored the following specific objectives relating to CPS immunization:

- to assess the life-cycle stage-specificity of CPS-induced protection (Chapter 6)
- to determine the safety and tolerability of intradermal administration of cryopreserved sporozoites to volunteers taking chloroquine chemoprophylaxis, and its protective efficacy against infectious mosquito bites (Chapters 7 & 8)

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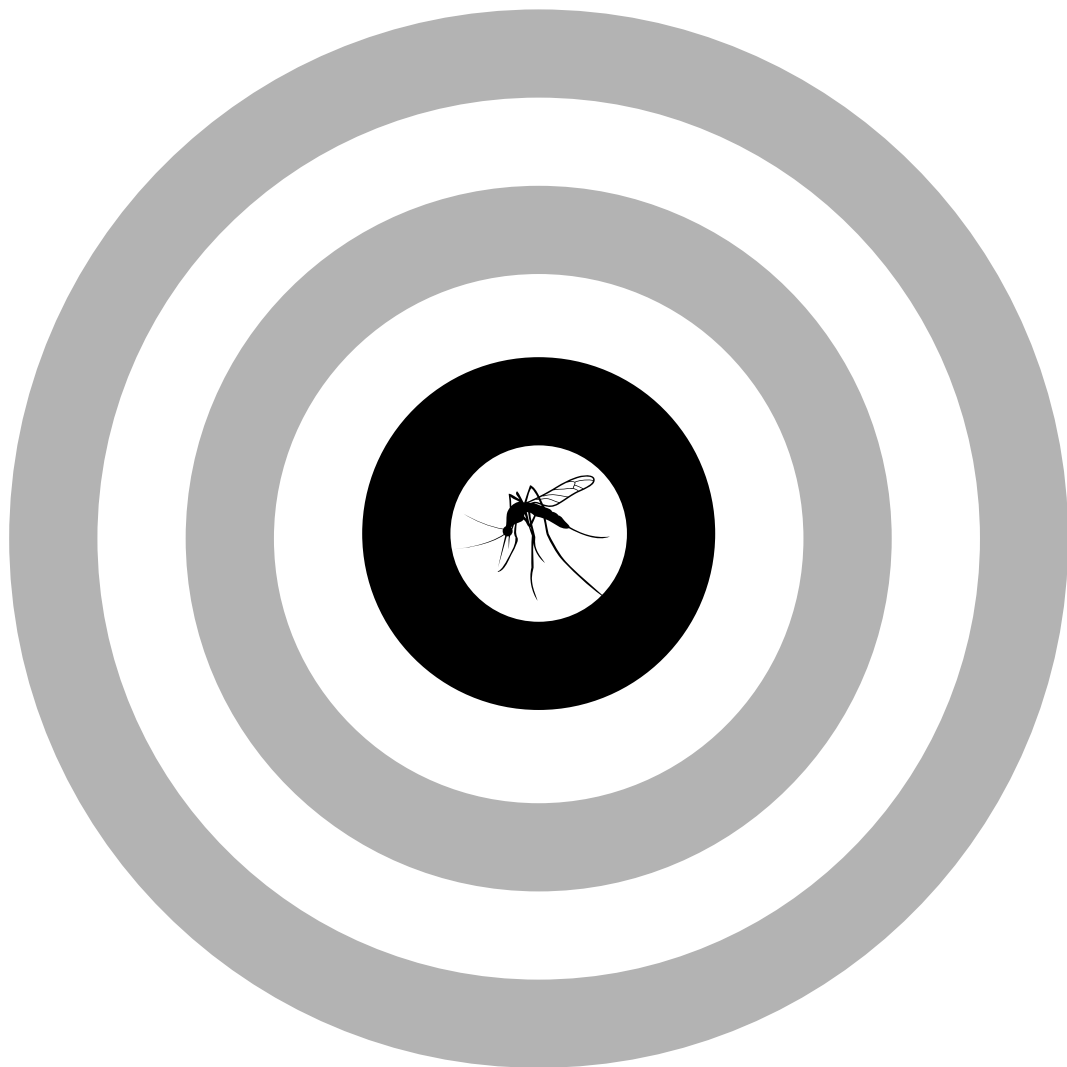
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SECTION A

**IMPROVING ACCESS
AND ACCURACY OF DIAGNOSIS USING
MALARIA RAPID DIAGNOSTIC TESTS**



CHAPTER 2

MALARIA DIAGNOSTIC TESTING AND TREATMENT PRACTICES IN THREE DIFFERENT *PLASMODIUM FALCIPARUM* TRANSMISSION SETTINGS IN TANZANIA: BEFORE AND AFTER A GOVERNMENT POLICY CHANGE

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Abstract

Introduction

Patterns of decreasing malaria transmission intensity make presumptive treatment of malaria an unjustifiable approach in many African settings. The controlled use of antimalarials after laboratory confirmed diagnosis is preferable in low endemic areas. Diagnosis may be facilitated by malaria rapid diagnostic tests (RDTs). In this study, the impact of a government policy change, comprising the provision of RDTs and advice to restrict antimalarial treatment to RDT-positive individuals, was assessed by describing diagnostic behaviour and treatment decision-making in febrile outpatients <10 years of age in three hospitals in the Kagera and Mwanza Region in northern Tanzania.

Methods

Prospective data from Biharamulo and Rubya Designated District Hospital (DDH) were collected before and after policy change, in Sumve DDH no new policy was implemented. Diagnosis of malaria was confirmed by RDT; transmission intensity was evaluated by a serological marker of malaria exposure in hospital attendees.

Results

Prior to policy change, there was no evident association between the actual level of transmission intensity and drug-prescribing behaviour. After policy change, there was a substantial decrease in antimalarial prescription and an increase in prescription of antibiotics. The proportion of parasite-negative individuals who received antimalarials decreased from 89.1% (244/274) to 38.7% (46/119) in Biharamulo and from 76.9% (190/247) to 10.0% (48/479) in Rubya after policy change.

Discussion

This study shows that an official policy change, where RDTs were provided and health care providers were advised to adhere to RDT results in prescribing drugs can be followed by more rational drug-prescribing behaviour. The current findings are promising for improving treatment policy in Tanzanian hospitals.

Introduction

Malaria accounts for more than 800,000 deaths a year worldwide [1], of which 90% are in young African children [2]. It is the leading cause of health service attendance in Tanzania and comprises ~40% of all morbidity in Tanzanian outpatients below 5 years of age [3]. This poses a considerable burden on health systems.

Since the 1980s, malaria control has incorporated presumptive treatment of fever with antimalarials, treating all febrile episodes suspected of malaria with a full therapeutic dose of antimalarials, chloroquine (CQ, until 2001 in Tanzania [4]) or sulphadoxine-pyrimethamine (SP, until 2007 [5]). This strategy was implemented to effectively treat true cases of malaria and may also have had beneficial effects on malaria transmission by effectively providing chemoprophylaxis to children in malaria endemic areas [6]. However, presumptive treatment may no longer be a justifiable approach in all settings now it is realized that many parts of Africa are characterized by low malaria transmission intensity [7,8] and transmission intensity is decreasing in many areas in sub-Saharan Africa [9-12]. In such settings, presumptive treatment will lead to substantial overtreatment [8], which is particularly important now the relatively expensive artemisinin combination therapy (ACT) is endorsed as first-line treatment [13-15]. Besides expenses, such practice will lead to a reduced susceptibility of parasites to ACT because of the uncontrolled use of artemisinins [16]. It is important that diagnosis is established before ACT is prescribed.

Microscopy is the gold standard method for diagnosis of malaria, but the provision of microscopic facilities may not always result in accurate diagnosis [2,8,14,17]. Malaria rapid diagnostic testing (RDT) forms an attractive alternative to routine microscopy with good sensitivity and specificity profiles [1,18] and more objective results. Wide-scale implementation of RDTs may, therefore, improve malaria diagnosis. This was acknowledged by the Tanzanian Ministry of Health and Social Welfare. The Kagera Region was the first in Tanzania where a policy change was implemented as a pilot to tackle the problem of malaria overdiagnosis. Malaria rapid diagnostic testing replaced routine microscopy as the main diagnostic tool and clinicians were advised to restrict treatment to RDT-positive individuals only.

Here, the impact of the policy change was assessed by describing diagnostic behaviour and treatment decision-making in febrile outpatients under 10 years of age in three hospitals in the Kagera and Mwanza Region in Tanzania. Transmission intensity in these areas, all nearby Lake Victoria, was assessed by serological markers of malaria exposure.

Methods

Study site

In this study, common practices in fever diagnosis in the outpatient departments were described for three designated district hospitals (DDH) in northern Tanzania: Biharamulo DDH and Rubya DDH, both situated in the Kagera Region, and Sumve DDH, situated in the

Mwanza Region. Biharamulo is situated at 1,490m, Rubya at >1,500m and Sumve at 1,100m. The transmission intensity of the catchment areas is suspected to be different based on their distance to Lake Victoria and altitude [19]. However, no formal assessments of transmission intensity have been reported.

Health care at these outpatient departments was largely provided by clinical officers with two to three years' clinical training. Pneumonia, HIV, tuberculosis, gastrointestinal infections, various parasite infections and malaria are common conditions in outpatients according to hospital records. Malaria transmission is related to the long rains in March–April and the less reliable short rains in October–November. The study was approved by the Kilimanjaro Christian Medical Centre Research Ethics Committee. Written informed consent was obtained from parents or caretakers of all participants.

Prospective data before and after policy change

Data were prospectively gathered from all patients under the age of 10 years presenting at the outpatient department of Sumve DDH with reported or measured fever in the months September 2009–January 2010. In Biharamulo DDH and Rubya DDH, identical data were collected in September–October 2009. This is the period before the policy change when microscopy was the only available tool to diagnose malaria. At the end of October 2009, the Kagera Region was the first region in Tanzania where a policy change was implemented by the National Malaria Control Programme of the Ministry of Health and Social Welfare. This policy change comprised the replacement of microscopy as the main diagnostic tool for malaria by RDTs (ICT Malaria Pf Cassette Test (ML01), ICT Diagnostics) with a sensitivity of 82% at parasite densities of 200 parasites/ μ L, of 97% for parasite densities >2,000 parasites/ μ L and a false positivity rate of ~1% [20]. Clinical officers were now strictly advised to only treat RDT-positive individuals with antimalarials; the advice was to look for alternative diagnoses in RDT-negative individuals. The medical officers in charge received training from government officials about the implementation of the policy change. After this, each medical officer in charge introduced the policy change during a two-day workshop to the clinical officers. To evaluate the short-term impact of this policy change, data were gathered for a second period in Biharamulo DDH (January 2010–February 2010) and Rubya DDH (October 2009–February 2010).

The same information was collected before and after policy change. The following information was collected in a questionnaire: age, gender, month attending the hospital, resident or no resident of the area in which the hospital was situated, bed net use, use of medication in the previous 2 weeks, axillary temperature, clinical diagnosis made by a clinical officer, request for blood slide, result of blood slide, treatment given in the hospital, and result of RDT. In Biharamulo and Rubya an additional question 'request for RDT' was added in the second period. Axillary temperature was measured with digital thermometers; blood slides were prepared according to hospital routine, stained with 10% Giemsa solution and read by a single experienced microscopist.

Before the policy change, a finger-prick blood sample was taken for malaria parasite detection by RDT (Paracheck Pf Rapid test, Orchid Biomedical Systems) in each patient enrolled for an objective assessment of infection with *Plasmodium falciparum*. The RDT was not part of hospital routine, but was used to facilitate objective diagnosis for the current study. The RDT was used according to the manufacturer's instructions and is based on the detection of histidine-rich protein 2 (HRP2). This RDT has a sensitivity of 75% at parasite densities of 200 parasites/ μL , of 100% for parasite densities $>2,000$ parasites/ μL and a false positivity rate of $\sim 7\%$ [20]. The results of the RDTs were available for clinical officers for diagnosis.

Transmission intensity

For an objective assessment of exposure to malaria, serum samples were collected using a single finger prick ($\sim 20 \mu\text{L}$) from all individuals, both adults and children, attending these three hospitals in Northern Tanzania in November 2009. This group included patients, people coming for routine follow-up visits and people accompanying patients. This sampling method has previously been validated to obtain a selection of samples representative of the general population in the hospital catchment areas [21]. Information was collected on age, gender, area of residency, bed net use, and use of medication in the previous 2 weeks.

Assessment of malaria exposure by enzyme-linked immunosorbent assay (ELISA)

Serum was eluted from filter papers as described by Corran et al. [22]. Immunoglobulin G antibodies against blood-stage antigens were detected by indirect ELISA, as previously described in detail [22,23] using recombinant apical membrane antigen 1 (AMA-1). AMA-1 was suggested as a suitable antigen for areas of moderate or low transmission intensity because of its high immunogenicity that results in saturating responses at high endemicity but a good discriminating power at low endemicity [24]. To generate an OD cut-off value above which samples were deemed antibody positive, the distribution of OD values was fitted as the sum of two Gaussian distributions (assuming a narrow distribution of seronegatives and a broader distribution of seropositives) using maximum likelihood methods [22].

Data analysis

Statistical analyses of data were performed using SPSS version 18.0.0 and Stata 9.2 (Stata Corp, College Station TX, USA). Categorical variables were compared between groups by the Pearson's chi-squared test or Fisher's exact test; odds ratios (OR) with 95% confidence intervals (95% CI) were calculated. The main outcomes of the analyses were the proportion of individuals treated with antimalarials despite a negative RDT and the proportion of individuals who did not receive antimalarials despite a positive RDT. AMA-1 ELISA data were used to generate an age-seroprevalence plot. OD values were expressed as percentage

of the positive control (normalized OD). The annual seroconversion rate (SCR), λ was estimated by fitting a simple model of the acquisition and loss of antibodies to the age-specific prevalence of the antibodies using maximum likelihood methods assuming a binomial distribution [23]. If visual examination of SCR suggested it was not uniform over the whole population (i.e. there was an obvious step in the age-seroprevalence data), models allowing for two forces of infection profile and profile likelihood plots were run to determine when the most likely time for change in transmission intensity occurred [21,25]. These resulted in a predicted time of change in transmission, which was incorporated in the catalytic model to generate estimates for previous and current SRC. Models allowing for two forces of infection were preferred if the fit compared to the single force model was significantly better by likelihood ratio test at a $P < 0.05$.

Results

Collected data

From September 2009–February 2010, a total of 1,608 outpatients below 10 years of age presenting with (reported) fever were included. Age, reported bed net use and socio-demographic factors were similar between the three clinics (Table 1). The proportion of individuals with current fever was lowest in Biharamulo.

Malaria case management before and after malaria treatment policy change

Diagnostic behaviour and clinical management in Rubya DDH are summarized in Figures 1 and 2; the same information for Biharamulo DDH and Sumve DDH is shown in the Supporting Information (SI) Figure 1, SI Figure 2 and SI Figure 3. Treatment prescribing behaviour before and after the policy change in relation to parasite prevalence is shown in Table 2 for all three clinics. There was a striking difference in RDT parasite prevalence between the three clinics with the lowest parasite prevalence by RDT in Rubya (1–4%) and the highest in Sumve (42%, 152/362). Although the majority of RDT-positive individuals received antimalarial treatment, in Biharamulo DDH, 5 malaria-positive individuals did not receive antimalarial treatment before policy change (8%, 5/67) compared to 2 (25%, 2/8) after policy change. In the other health centres, a positive RDT was followed by antimalarial treatment in all instances. In all clinics a substantial proportion of slide-negative patients received antimalarial treatment before the policy change (Sumve 67%, Biharamulo 100%, Rubya 81.1%) and a substantial proportion of RDT negative patients received antimalarial treatment (Sumve 68.1%, Biharamulo 89.1%, Rubya 76.9%). After the policy change this decreased to 38.7% in Biharamulo DDH and 10.0% in Rubya DDH.

There was also a striking decrease in presumptive treatment of reported fever with antimalarials. In Biharamulo DDH 89.0% (284/319) of patients without a slide taken was treated with antimalarials before the policy change compared to 27.0% (27/100) of patients

Table 1 Characteristics of the study population

	Sumve DDH		Biharamulo DDH		Rubya DDH	
			Before policy change	After policy change	Before policy change	After policy change
Study period	Sep '09–Jan '10		Sep '09–Oct '09	Jan '10–Feb '10	Sep '09–Oct '09	Oct '09–Feb '10
Number of observations	362		360	135	250	501
Age, months, median (IQR)	15.0 (9.0–24.0)		15.0 (9.0–27.0)	14.5 (8.0–25.8)	12.0 (8.8–24.0)	14.0 (8.0–24.0)
Temperature						
Mean (sd)	37.5 (1.0)		37.0 (1.0)	37.4 (1.0)	37.8 (1.0)	37.7 (1.1)
Fever ($\geq 37.8^{\circ}\text{C}$), % (n/N)	39.2 (135/344)		19.1 (68/356)	29.2 (38/130)	48.4 (121/250)	46.9 (235/501)
Reported use of bed net, % (n/N)	95.8 (340/355)		98.9 (356/360)	95.5 (128/134)	95.6 (239/250)	95.2 (477/501)
Reported antimalarial use in preceding two weeks, % (n/N)	26.5 (95/358)		74.0 (361/488)		6.4 (48/751)	
Number of serum samples collected	308		300		320	

IQR, interquartile range.

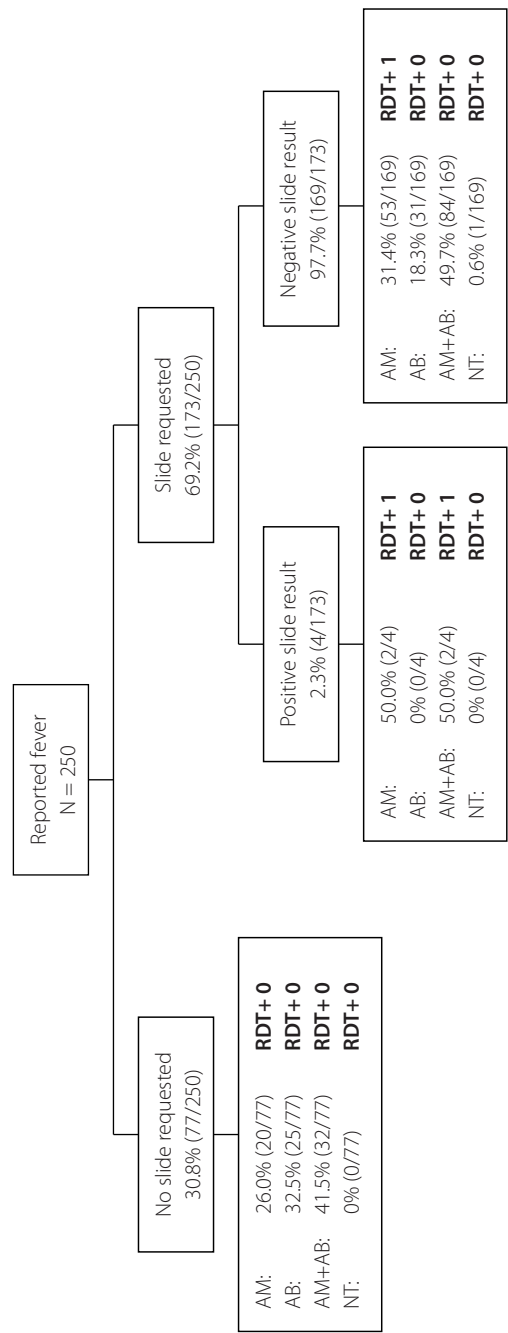


Figure 1 Rubya DDH before policy change

RDT, malaria rapid diagnostic test; AM, antimalarial treatment given; AB, antibiotic treatment given; NT, no treatment installed

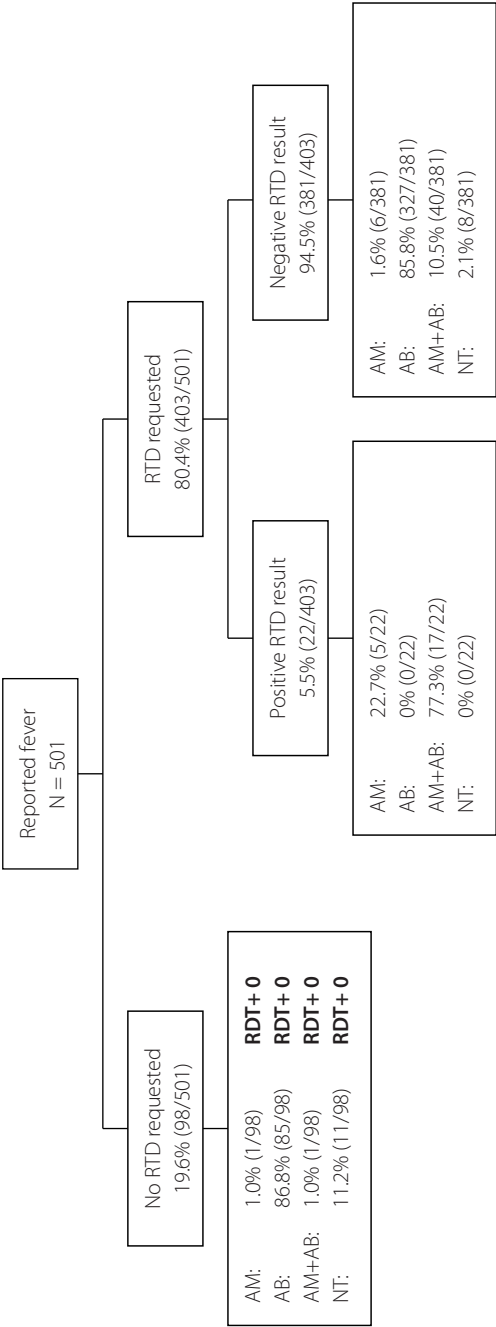


Figure 2 Rubya DDH after policy change

RTD, malaria rapid diagnostic test; AM, antimalarial treatment given; AB, antibiotic treatment given; NT, no treatment installed

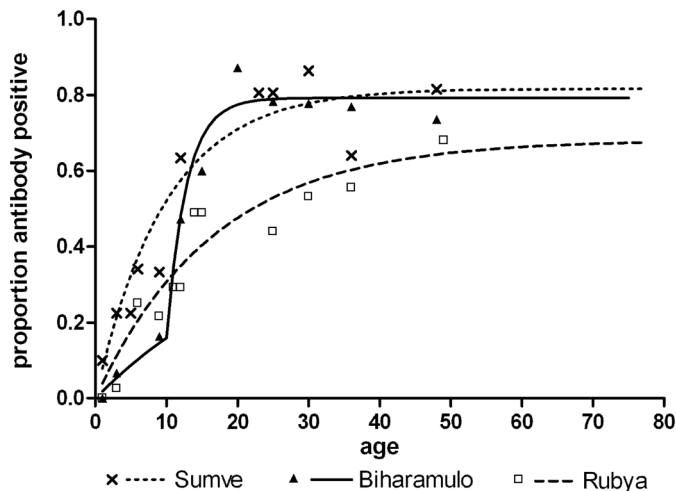


Figure 3 Age specific seroprevalence plots for apical membrane antigen 1 for Sumve, Biharamulo and Rubya

For Biharamulo two forces of infection were fitted, for the other two sites one force of infection fitted the data best. The seroconversion rate (SCR, λ) for Sumve was 0.082 (95% CI, 0.063–0.11). For Biharamulo the SCR was 0.34 (95% CI, 0.19–0.61) for the period up to 1999 (i.e. for individuals older than 10 years of age) but 0.019 (95% CI, 0.011–0.035) for the period 1999–2009. The SCR for Rubya was 0.041 (95% CI, 0.029–0.058).

without a RDT after the policy change. In Rubya DDH this proportion changed from 67.5% (52/77) to 2.0% (2/98), indicating presumptive treatment was largely abandoned after the policy change.

Management of non-malaria fevers before and after malaria treatment policy change

Together with the decrease in antimalarial prescription, there was an increase in the proportion of individuals who received antibiotics. These were mostly broad-spectrum antibiotics like co-trimoxazole, ciprofloxacin or amoxicillin. In RDT-negative patients in Biharamulo DDH 12.8% received antibiotics before and 59.7% after the policy change. In Rubya DDH the number increased from 70.0% to 94.6%.

Table 2 Treatment practices in relation to malaria parasite prevalence by malaria rapid diagnostic test

	Sumve DDH		Biharamulo DDH		Rubya DDH	
			Before policy change	After policy change	Before policy change	After policy change
Reported fever	362		360	135	250	501
Proportion RDT+, % (n/N)	42.0% (152/362)		19.6% (67/341)	6.3% (8/127)	1.2% (3/250)	4.4% (22/501)
Antimalarial treatment						
RDT+, % (n/N)*	100% (152/152)		92% (62/67)	75.0% (6/8)	100% (3/3)	100% (22/22)
RDT-, % (n/N)**	68.1% (143/210)		89.1% (244/274)	38.7% (46/119)	76.9% (190/247)	100% (48/479)
Antibiotic treatment						
RDT+, % (n/N) #	28.9% (44/152)		16.4% (11/67)	25.0% (2/8)	33.3% (1/3)	77.3% (17/22)
RDT-, % (n/N) ##	63.3% (133/210)		12.8% (35/274)	59.7% (71/119)	70.0% (173/247)	94.6% (453/479)

* proportion of RDT-positive individuals who received antimalarial treatment

** proportion of RDT-negative individuals who received antimalarial treatment

proportion of RDT-positive individuals who received antibiotics

proportion of RDT-negative individuals who received antibiotics

The estimated transmission intensity generated from seroprevalence data

From Biharamulo DDH, Rubya DDH, and Sumve DDH, a total of 298, 320 and 304 serum samples were successfully tested in the AMA-1 ELISA, respectively. The overall seroprevalence of AMA-1 antibodies was 52.0% (158/304), 40.3% (129/320), and 56.7% (169/298); there was a clear increase in seroprevalence with age in all sites (Figure 3). The seroconversion rate was highest in Sumve ($\lambda=0.082$; 95% CI, 0.063–0.11) and lowest in Rubya ($\lambda=0.041$; 95% CI, 0.029–0.058), in line with parasite prevalence rates by RDT. In Biharamulo, malaria transmission intensity appeared to have changed over time. While transmission intensity used to be intense ($\lambda=0.34$; 95% CI, 0.19–0.61), the current SCR was similar to that of Rubya ($\lambda=0.019$; 95% CI, 0.011–0.035), indicating low transmission intensity.

Discussion

In three study areas in the vicinity of Lake Victoria, a considerable overuse of antimalarials was seen prior to a new government policy to incorporate RDTs in the diagnostic process. There was no evident association between the actual level of transmission intensity and drug-prescribing behaviour across the sites: two thirds of the RDT-negative individuals received antimalarial drugs in the area of lowest endemicity. An official policy change, where RDTs were provided and health care providers were advised to adhere to RDT results in prescribing drugs was followed by lower antimalarial prescription rates and higher antibiotic prescription rates in parasite-negative children. This study indicates that an official policy change may reduce the overuse of antimalarials, resulting in a more rational drug-prescribing behaviour.

In this study, treatment policies are described in children with reported fever visiting outpatient departments of three district hospitals in regions of differing malaria transmission intensity. Baseline diagnostic behaviour was similar between hospitals and overtreatment, defined as antimalarial treatment of RDT-negative individuals, ranged from 68–90%, which is similar to previous studies [8,13,14]. Prior to policy change, these RDT results were provided to have an objective indicator of malaria infection for this study. The RDT was not requested by the responsible clinical officer who mostly based his or her diagnosis on clinical symptoms alone and microscopic results were often ignored in the final decision-making. Overtreatment was particularly common in Rubya and Biharamulo, areas where parasite prevalence by RDT and transmission intensity by serological markers of malaria exposure indicated low current levels of malaria transmission intensity. Like other recent studies [1,8,14], this study shows a discrepancy between the perceived and actual levels of transmission intensity. While there was no evidence for recent changes in transmission intensity in Rubya, transmission intensity appeared to have dropped in Biharamulo in the last decade. The reason for this change in transmission is unknown, but has been observed before in northern Tanzania [21].

Presumptive treatment of fevers with antimalarials may have a beneficial prophylactic effect that could also result in a reduction in malaria transmission by reducing the human infectious reservoir [6]. However, in a setting like Rubya where transmission intensity is low, this beneficial effect is unlikely. The diagnosis of malaria and according treatment may simply be a 'convenient' clinical strategy avoiding the more complicated search for other causes of the presenting illness [26]. Treatment of all febrile episodes as malaria is likely to result in underdiagnosis of other fever-causing disorders such as childhood pneumonia [2]. In addition, there are financial implications. Overtreatment will often be with the highly effective, but expensive, artemether-lumefantrine (AL). In the current study, all malaria episodes were treated with AL. AL and other ACTs are 10-times more expensive than previously used drugs as sulphadoxine-pyrimethamine [27,28] making reliable diagnosis crucial for cost-effective use [29]. Importantly, there is concern for a reduced susceptibility of *P. falciparum* parasites for ACT [30] and the spread of parasites with reduced susceptibility to ACT may be enhanced by irrational drug use [31]. Reports on allelic selection after AL overuse [32] provide additional warnings against overuse of ACT.

Microscopic examination of a blood smear is the gold standard method for the diagnosis of malaria, but even if microscopy is used, overdiagnosis of malaria is common [17]. In Sumve DDH and Rubya DDH clinicians frequently requested blood slides, but also frequently ignored negative results. In these hospitals up to 81% of the slide-negative individuals were treated with antimalarials. In Biharamulo DDH 79% of the children were treated with antimalarials while no lab-confirmation was sought (i.e. no slide was requested). The Tanzanian Ministry of Health and Social Welfare implemented a policy change in the Kagera Region to tackle malaria overdiagnosis and overtreatment by replacing routine microscopy with RDTs as main diagnostic tool. Although RDTs may not be 100% sensitive in detecting (low-density) malaria infections [1], a prospective study found that restricting antimalarials to RDT-positive individuals is a safe strategy in low endemic areas that does not lead to excess mortality due to false-negative results [33]. RDT results are objective and can also be confirmed by the person responsible for the clinical decision-making who may not be confident with microscopy. The two different RDTs used in this study are both based on HRP-2 for parasite detection and have similar sensitivity; we do not expect marginal differences in RDT sensitivity to have influenced the treatment-prescribing behaviour. The results of this study indicate an evident short-term impact on diagnostic behaviour and treatment decisions, unlike results from a previous study in Zambia [34]. In Rubya DDH, the proportion of individuals receiving antimalarials despite negative RDTs was reduced 7-fold. This indicates that clinicians appeared willing to trust the RDT result in their decision-making, at least shortly after receiving clear instructions from the Tanzanian Ministry of Health. Presumptive treatment was not completely abandoned but decreased substantially. It is encouraging that antibiotic prescription increased in RDT-negative individuals, indicating that alternative diagnoses and treatment options are considered.

The current study was purely observational and no attempts were made to change the clinical decision-making process during the study. The new government guidelines were explained by the medical officers in charge and information on the sensitivity of RDTs was provided. Although an effect of the present study cannot be ruled out completely, similar observational studies showed no positive effect on diagnostic and drug-prescribing behaviour [8,14]. Therefore, the impact of the current observational study on treatment practices will have been negligible compared to the new guidelines provided by the government. There are conditions for the policy change to work and keep working. First of all, the hospital staff must be well-trained and confident regarding the new policy and new diagnostic test and clear guidelines should also be provided on treatment policies for RDT-negative individuals. Secondly, RDTs must be available at all times, as well as antimalarial drugs and antibiotics. In Rubya DDH, RDTs provided by the Tanzanian government were out of stock in the beginning of February 2010. This is also seen in other countries [34]. Consequently, this brings the risk of hospital staff returning to their former diagnostic and drug-prescribing behaviour.

Conclusion

This study shows promising data on decrease of antimalarial drug-prescription. Larger prospective studies are needed to confirm the current findings, to determine the impact of the new policy on morbidity and mortality and to assess the long-term impact of the described policy change. The current findings on rational drug-prescribing behaviour in Tanzanian hospitals are promising.

Acknowledgements

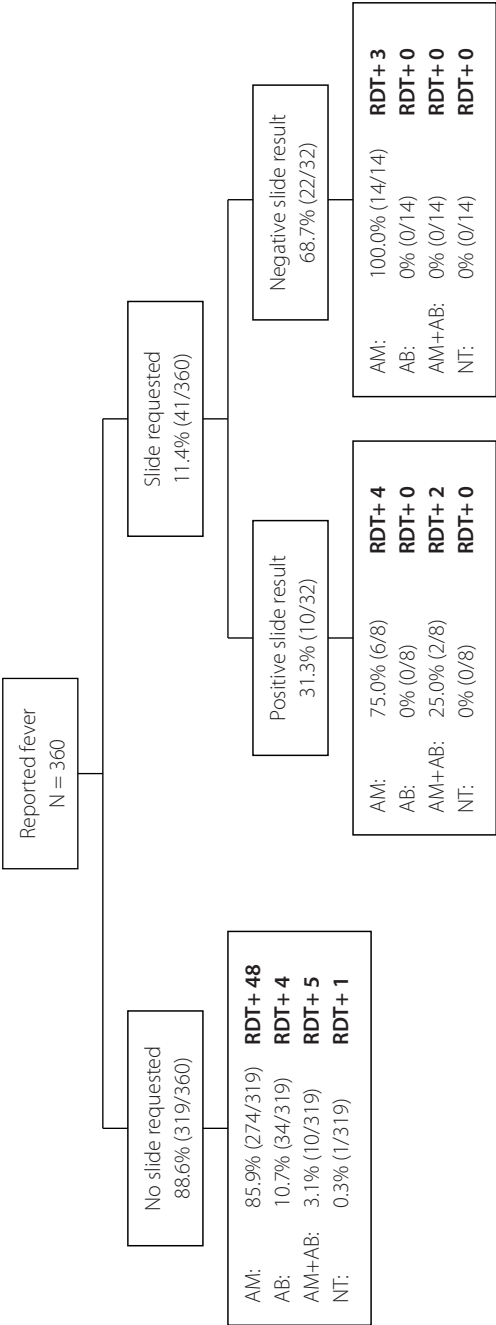
We'd like to thank the hospital attendees for their participation in the study. Special thanks go out to all participating medical officers and clinical officers from Biharamulo DDH, Rubya DDH and Sumve DDH, and all participating medical students. Furthermore, we would like to thank H. van Asten from the Nijmegen Institute for International Health from the Radboud University Nijmegen.

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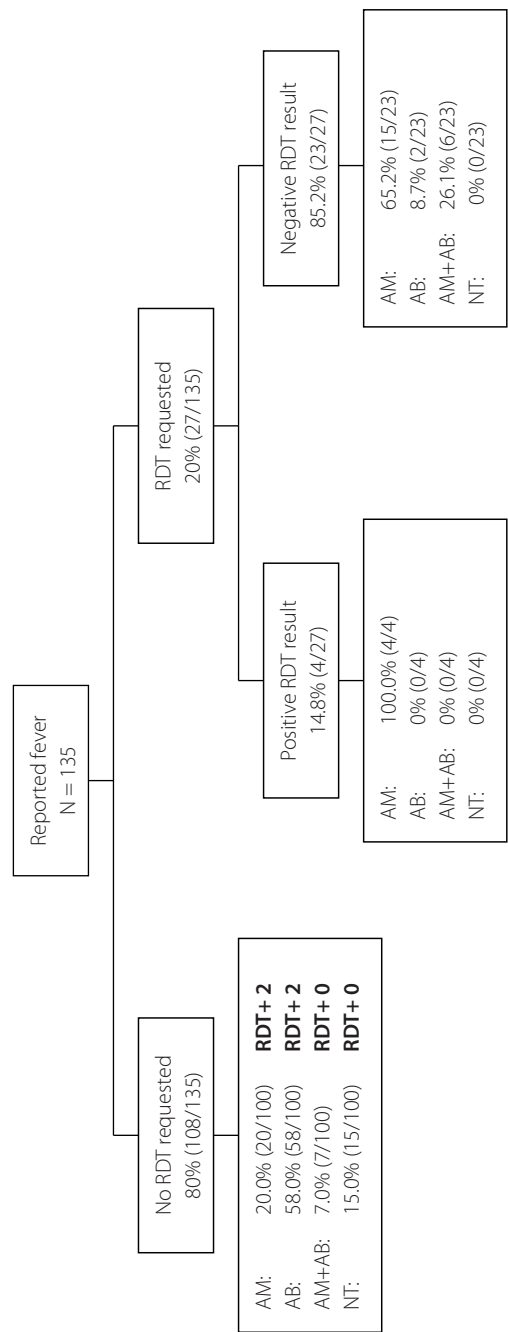
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Supporting Information



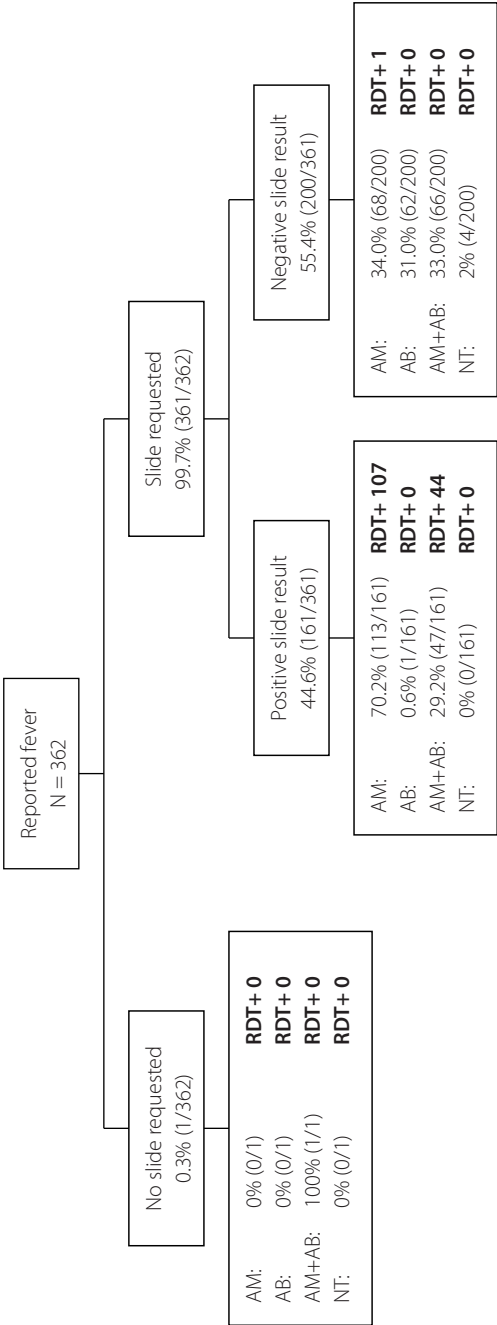
SI Figure 1 Biharamulo DDH before policy change

RDT, malaria rapid diagnostic test; AM, antimalarial treatment given; AB, antibiotic treatment given; NT, no treatment installed



SI Figure 2 Biharamulo DDH after policy change

RDT, malaria rapid diagnostic test; AM, antimalarial treatment given; AB, antibiotic treatment given; NT, no treatment installed



SI Figure 3 Sumve DDH (no policy change)

RDT, malaria rapid diagnostic test; AM, antimalarial treatment given; AB, antibiotic treatment given; NT, no treatment installed

CHAPTER 3

SCALE-UP OF MALARIA RAPID DIAGNOSTIC TESTS AND ARTEMISININ-BASED COMBINATION THERAPY: CHALLENGES AND PERSPECTIVES IN SUB-SAHARAN AFRICA

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Abstract

- Scaling up and sustaining access to malaria diagnosis and treatment in all public sector, for-profit, and informal health facilities across sub-Saharan Africa is central to current global strategies for malaria control and elimination.
- The use of malaria rapid diagnostic tests (RDTs) aims to eliminate reliance on signs and symptoms to diagnose and treat malaria but evidence shows health workers do not always test the right patients, nor provide treatment based on the results of the test.
- Expanding access to malaria RDTs on the scale needed to achieve universal coverage requires retraining of public, private, and retail sector providers as well as sustained supplies and quality assurance.
- Barriers to rational use of tests and drugs may be overcome through appropriate policy design for the local health service setting, which addresses health worker practice and patient perceptions.
- Innovative methods have been used to increase access to the most effective antimalarial drugs in the last five years, but these efforts will be incomplete and unsustainable without similar efforts to incorporate RDTs into practice.

Introduction

An estimated 627,000 malaria deaths occurred in 2012, mostly in African children and many of them preventable with prompt diagnosis and treatment [1]. Access to diagnosis remains poor—in half of endemic African countries, over 80% of malaria treatments are applied without diagnostic testing [2]. Improving diagnosis and treatment of malaria will improve treatment outcomes, rationalize health care costs by reducing drug consumption [3], minimize drug pressure that can contribute to resistance [4,5], and assist in monitoring disease trends [2].

In April 2012, the World Health Organization's (WHO) Global Malaria Programme launched a highly ambitious new initiative: *T3: Test. Treat. Track* [1,2]. T3 aims to address the widespread problem of poor access to diagnostic testing and antimalarial treatment, and to enhance case-reporting. It sets a target of universal access to diagnostic testing in the public and private health care sector by 2015 [1,2]. Achieving this goal will centre on the use of malaria rapid diagnostic tests (RDTs).

In this Policy Forum article we examine the operational challenges to implementing the T3 strategy of scaling up and maintaining RDT coverage. We identify gaps in planning for at-scale implementation in policy design and implementation, the local health care setting, and the attitudes and demands of patients. While focussed on malaria diagnosis and treatment, the challenges illustrated here are not unique to malaria and may apply to health care provision across resource-poor settings.

Policy design and implementation

By 2012, 41 out of 44 endemic countries in the WHO African Region had adopted the policy of providing malaria diagnostic testing for all age groups before treatment [2]. RDT procurement increased worldwide from 45 million units in 2008 to 205 million in 2012 although supply remains far short of requirements [1,2]. In theory, the availability of reliable easy-to-use tests should result in a switch from presumptive treatment based on signs and symptoms alone, to parasite-based diagnosis and treatment based on test results. Diagnostic processes and treatment decisions are, however, often irrational and health staff do not always test the right patients, nor provide treatment based on the results [6-8].

RDTs will be introduced in health facilities and among community health workers (CHWs) who work at local levels. To translate the change in policy to a change in routine practice where tests are appropriately used by providers, unambiguous messages and guidelines that are adapted to the local context are needed [6,9,10]. This targeted information must counter the widespread and long-held guidelines that promoted presumptive treatment of malaria in cases of fever [11]. Appropriate information and training will improve implementation at the community level [12]. Recent evidence shows that CHWs reliably provided Integrated Management of Childhood Illnesses to children

after training and incorporation of RDTs into the algorithm [13,14]. In one study, malaria and pneumonia were appropriately classified in 94%–100% of children, and supply management of medications and RDTs was excellent [13]. Replicating these effects outside the trial setting requires national level training to ensure safety and quality of services.

Mobilising sufficient resources for the training and monitoring required to sustain the new policy is the key to success. A reliable system for RDT delivery needs to include re-training of staff and consistent quality assurance at all levels. The quality of services is likely to wane over time and can be aggravated by high staff turnover, which occurs in many health service settings. Ensuring programme quality and sustainability therefore requires constant rolling interventions and local evidence for the best models of implementation.

The local health care setting

In the local health care setting, two problems persist: firstly, parasite-based testing is generally unavailable [1,2] with treatment decisions based on clinical signs and symptoms that are neither sensitive nor specific [15]; and secondly, if tests are available, health workers often do not apply treatment according to the result of the test [10,16–18]. Both situations result in extensive overuse of antimalarial drugs, especially in low transmission settings [19,20].

When RDTs are introduced in presumptive treatment settings significant reductions in the overprescription of antimalarials have been seen in almost all studies published (Supporting Information Table 1). However, when they are introduced in settings that have used microscopic examination of blood smears, the advantages of RDTs are harder to define. Substantial numbers of patients may still be treated with an antimalarial drug despite a negative RDT or blood smear result, so the evidence of any clinical advantage of RDTs over microscopy is unclear in some settings (Supporting Information Table 2).

Often, the irrational use of tests and drugs is based on perceived shortcomings of the tests. A common concern amongst health staff is that negative tests do not definitively rule out malaria [21], but trials that withheld antimalarials in febrile children with negative test results have shown no additional malaria risk to patients in moderate-to-high transmission settings. In one trial in Uganda, 13/1,602 (0.8%) blood smear-negative patients who were not given antimalarial drugs developed clinical malaria over 7 days of follow-up and all 13 were detected by the health service and treated [22]. Similar findings were seen in Tanzania (3/603 [0.5%] of RDT-negative patients developed malaria within 7 days) [23]. These studies indicate that withholding antimalarial therapy in febrile children with negative test results is likely to be safe and results in a considerable reduction in antimalarial drug consumption.

Improvements in antimalarial prescription often coincide with increases in prescription of antibiotics amongst test-negative patients. All studies where antimalarial prescription rates were reduced in malaria-negative patients show an increase in antibiotic prescriptions

(Tables S1 and S2) [16,19,24-26]. There is little data on the spectrum of infections in patients presenting with symptoms of suspected malaria but most of these are probably self-limiting [23,27], and evidence that supports the prevailing practice of widespread antibiotic use in malaria-negative patients is lacking.

Identifying patients at risk of progressing to severe disease in which antibiotic treatment and/or referral would have a clinical advantage, while withholding antibiotic treatment in other patients, is a considerable challenge. Affordable rapid diagnostics for bacterial infections or markers of severe infections would support the rational prescription of both antimalarials and antibiotics.

Patient load and malaria diagnosis

A high patient load in many clinics creates challenges in implementing new policies and motivating staff [28,29]. In Tanzania, health workers identified high patient load and shortage of staff as key factors that hindered use of RDTs [28]. Most staff felt RDTs placed additional strain on normal operations and believed more staff were needed to conduct the tests [28]. Although these considerations apply to all diagnostic procedures and are not unique to RDTs, understanding the realities of routine practice is required because introducing extra staff into facilities will have an impact on cost.

Sustained supply of RDTs in public and private sectors

Sustaining the supply of RDTs is a substantial challenge. In rural areas, where access to services is often low but demand for services may be highest [1], drug stock-outs are common [30,31] and supply is one of the biggest challenges facing the health system. The T3 recommendations imply that a constant supply of both artemisinin-based combination therapies (ACTs) and RDTs is needed. The shelf-life and performance of both diagnostics and drugs depends on their storage conditions; RDTs are degraded by high temperatures and humidity and the entire supply chain must ensure that RDTs remain within manufacturers' recommended limits. WHO testing of a range of commercially available RDTs demonstrated consistent detection of malaria at tropical temperatures [21], but actual field data on storage conditions affecting RDT stability are scarce.

The private for-profit sector plays an important role in delivering services across most of Africa and the majority of suspected malaria episodes are initially treated by private health workers [32,33]. Data from a limited number of countries suggest neither microscopy nor RDTs have penetrated the private health care sector [1,34] but more than 50% of patients purchase drugs from unregistered shops and peddlers [32,33]. This occurs especially amongst lower income groups [35]. Improving diagnostic and treatment practices in the private sector could have a substantial impact on access to diagnosis before treatment but models of implementation have yet to be fully assessed in operational trials [35,36].

Affordability and cost-effectiveness of RDT-based diagnosis

To improve access to drugs in sub-Saharan Africa, the *Affordable Medicines Facility - malaria* provided subsidised ACT drugs in a multi-country pilot [37]. This study demonstrated improved access and market share of ACTs in 5 out of 7 pilot countries driven mainly by improvements in the private for-profit sector [38]. In 2012, 331 million courses of ACTs were procured by the public and private sectors in endemic countries, up from 182 million in 2010 [1]. Although the pilot rapidly improved availability, affordability, and market share of quality-assured ACTs at the point of use, no equivalent increase in RDTs has been observed [1,38]. As diagnosis is seldom available and ACT orders are more than double that of RDTs, overtreatment is likely to be common in retail outlets. ACTs are approximately 10 times more costly than previously used monotherapies [19,31] so the use of RDTs prior to treatment may improve cost-effectiveness. Data from a willingness-to-pay study in private drug shops in Uganda indicated that there was a demand for RDTs in the private sector but this was far below the price of delivery [39]. Subsidised supply of RDTs, similar to the ACTs subsidy, should be assessed to examine the impact on the uptake of RDTs in the private retail sector.

In high and very high transmission areas, presumptive treatment has cost-effectiveness advantages given the imperfect sensitivity of tests under field conditions [3]. RDTs in settings with up to 62% *Plasmodium falciparum* prevalence were cost-effective compared to presumptive treatment, assuming that prescribers adhered fully to test results [31]. When treatment is consistent with the results of a test, cost savings of between 50% and 100% can be achieved compared with presumptive treatment [3]. Conversely, if treatment is inconsistent with the result of the test, cost-effectiveness is reduced, an association that varies with the malaria transmission setting [3,31]. Other factors that can reduce cost-effectiveness are stock-outs, poor accuracy of RDTs, and poor quality assurance for drugs and diagnostics [31].

In low-endemic settings, RDTs and microscopy remain attractive compared to presumptive treatment even when there is poor adherence to negative test results [3]. RDTs can be more cost-effective than microscopy because they are more accurate under real-life conditions [31] and continuous (re-)training of microscopists is particularly important if fewer malaria-positive slides with low parasite levels are encountered in low-endemic settings. Despite these advantages of RDTs over presumptive treatment, adherence to microscopy and RDT test results remains a key factor for cost-effective diagnosis and treatment [3,40].

Malaria diagnosis in elimination programmes

Currently available RDTs will not detect all infections with low parasite loads. These submicroscopic infections frequently occur in low-endemic areas [41], are probably not associated with clinical risks [42], but do play a role in onward malaria transmission [43]. Diagnostics with a sensitivity that is higher than currently available RDTs will be needed to

identify all malaria infections in elimination efforts [44]. Operational approaches may involve screening by RDT to identify geographic or demographic clusters of infections [45,46] that can be targeted following molecular diagnosis of infection or by focal mass drug administration [47,48].

Attitudes and demands of patients

Patients can influence the diagnostic and treatment practices of health workers [7,8] and patient pressure on providers contributes to overtreatment [7]. There is a persistent perception that all fever episodes in malaria endemic areas are due to malaria [49] and, until recently, a global policy of presumptive treatment for malaria in cases of fever has been in place [2]. These factors have created entrenched demand for malaria treatment without first testing for malaria [29,50,51]. Efforts to change demands to promote malaria testing are particularly important in the private and informal sector, where few patients presently receive a diagnostic test. A change in public perceptions brought about by effective communication is needed to widen demand for testing before treatment.

Conclusions

Meeting the global target of universal coverage with parasite-based diagnosis by 2015 is a huge undertaking requiring sufficient resources. The cost-effectiveness of the intervention will hinge on the accurate use of RDTs in guiding treatment. Probably the biggest challenge in RDT implementation will be to provide adequate and sustained supplies of RDTs and appropriate training to all health workers in endemic areas. With increased access to malaria diagnosis, there will also be increased use of antibiotics, and interventions to guard against even greater overuse are needed to prevent worsening antimicrobial resistance. The *Affordable Medicines Facility - malaria* initiative demonstrated that large increases in access to ACTs were possible. Increasing access to RDTs is equally important. ACTs and RDTs should be seen as a package to improve management of febrile cases, and improving access to both of these in the public and private sectors has the potential to provide valuable returns.

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Supporting information

SI Table 1 Patients treated with antimalarials and antibiotics in studies comparing clinical diagnosis with malaria rapid diagnostic tests

Study, year	Country	Study design	Treatment with antimalarials
			Clinical diagnosis
			% (95% CI), n/N
Williams et al., 2008	Tanzania	Pilot implementation study	61.3 (57.4–65.3), 365/595
Msellem et al., 2009	Tanzania	Cross-over validation trial	85.3 (82.9–87.6), 752/882
Bisoffi et al., 2009	Burkina Faso	Randomized, multi-centre, open clinical trial	87.2 (85.3–89.2), 969/1111
Kyabayinze et al., 2010	Uganda	Quasi-experimental implementation study	54.3 (53.9–54.7), 29117/53629
Ansah et al., 2010	Ghana	Randomized controlled open label trial	92.7 (91.5–94.0), 1598/1723
Chinkhumba et al., 2010	Malawi	Cross-sectional survey	NR
Masanja et al., 2010	Tanzania	Observational study	NR
Yeboah-Antwi et al., 2010	Zambia	Cluster randomized controlled trial	99.1 (98.7–99.5), 2066/2084
Chanda et al., 2011	Zambia	Prospective pilot study	NR
Mubi et al., 2011	Tanzania	Randomized cross-over trial	96.5 (95.6–97.5), 1422/1473
Ishengoma et al., 2011	Tanzania	Longitudinal study	98.6 (98.3–98.9), 4448/4511
Uzochukwu et al., 2011	Nigeria	Cross-sectional study	NR
Mukanga et al., 2012	Burkina Faso	Open cluster randomized trial	99.8 (99.5–100.0), 575/576
Mukanga et al., 2012	Ghana	Open cluster randomized trial	95.3 (93.5–97.0), 563/591
Mukanga et al., 2012	Uganda	Open cluster randomized trial	100.0, 965/965
Kyabayinze et al., 2012	Uganda	Cross-sectional survey	60.1 (59.3–60.9), 8432/14024

RDT, malaria rapid diagnostic test; NR, not reported.

^a99% of RDT-positives received antimalarials, 30% of febrile RDT-negatives received antimalarials.

^b3 out of 704 RDT-negatives received antimalarials.

^cData of two districts.

^dAntibiotics not available as treatment.

Search strategy: on 24 April 2013 we searched the PubMed electronic database to locate relevant RDT-implementation studies from sub-Saharan Africa, focusing on intervention and observational studies of non-RDT versus RDT and pre-versus post-RDT implementation. We used the following combination of search terms:

Search #1: ("case management"[Text Word]) AND ("malaria"[Text Word]) AND ("rapid diagnostic test"[Text Word])

Search #2: ("treatment practices"[Text Word]) AND ("malaria"[Text Word]) AND ("rapid diagnostic test"[Text Word])

			Treatment with antibiotics	
RDT			Clinical diagnosis	RDT
% of RDT-positives	% of RDT-negatives			% of RDT-negatives
98.4 (97.7–99.0), 1329/1351	24.7 (22.3–27.2), 289/1168	NR	NR	NR
35.9 (33.0–38.9), 361/1005		26.6 (23.7–29.6), 235/882	37.0 (34.0–40.0), 372/1005 ^a	
97.7 (96.4–98.9), 543/556	81.0 (77.5–84.4), 400/494	54.8 (51.9–57.7), 609/1111	56.9 (52.5–61.2), 281/494	
32.9 (32.5–33.2), 24591/74784 ^a		NR	NR	
99.6 (99.1–100.0), 703/706	49.5 (46.4–52.5), 501/1013	22.3 (20.3–24.3), 384/1723	35.0 (32.1–38.0), 355/1013	
98.0 (97.1–98.9), 985/1005	57.9 (53.0–62.9), 223/385	NR	NR	
97.1 (96.7–97.6), 5331/5488	4.0 (3.4–4.5), 205/5162	NR	NR	
27.5 (24.7–30.3), 265/963 ^b		NR	NR	
100.0, 2318/2318	0.6, 13/NR and 0.0, NR ^c	NR	NR	
99.7 (99.3–100.0), 731/733	6.1 (4.3–7.8), 44/724	NR	NR	
31.8 (31.1–32.6), 4440/13942		NR	NR	
100.0, 92/92	74.1, (65.8–82.3) 80/108	NR	NR	
99.2 (98.4–100.0), 388/391	3.7 (0.5–6.9), 5/134	NR ^d	59.0 (50.6–67.3), 79/134	
100.0, 492/492	3.3 (0.0–6.9), 3/92	64.3 (60.4–68.2), 380/591	69.6 (60.2–79.0), 64/92	
99.9 (99.7–100.0), 856/857	7.6 (2.8–12.4), 9/118	NR ^d	59.3 (50.5–68.2), 70/118	
32.7 (32.0–33.5), 4892/14940		NR	NR	

Search #3: ("implementation"[Text Word]) AND ("malaria"[Text Word]) AND ("rapid diagnostic test"[Text Word])

Search #4: ("treatment outcome"[Text Word]) AND ("malaria"[Text Word]) AND ("rapid diagnostic test"[Text Word])

Search #5: ("adherence"[Text Word]) AND ("malaria"[Text Word]) AND ("rapid diagnostic test"[Text Word])

The search yielded 189 records from which 57 duplicates were removed. After screening the records for relevant studies, 106 records were excluded. Of the remaining 26 records, 5 records were excluded because these did not provide data on non-RDT versus RDT settings (Abdelgader et al. BMC Public Health 2012, 12:11; Mangham et al. Trop Med Int Health 2012, 17(3): 330-42; Manirakiza et al. BMC Public Health 2012, 12:482; Rowe et al. Mal J 2009, 8:275; Ukwaja et al. Ethiop J Health Sci 2010, 20(3):179-83). Results of the remaining 21 records are described here, showing studies performed in presumptive treatment (SI Table 1) and microscopy settings (SI Table 2), respectively.

SI Table 2 Patients treated with antimalarials and antibiotics in studies comparing microscopy with malaria rapid diagnostic tests

Study, year	Country	Study design	Treatment with antimalarials	
			Microscopy	
			% of slide positives (95% CI), n/N	% of slide-negatives
Hamer et al., 2007	Zambia	Cross-sectional, cluster sample survey	100.0, 64/64	58.4 (47.7–69.4), 45/77
Reyburn et al., 2007	Tanzania	Randomized trial	98.3 (96.3–100.0), 171/174	50.8 (47.7–53.8), 523/1030
Skarbinsky et al., 2009	Kenya	Pre-post cluster randomized controlled trial	59.2 (47.7–70.6), 42/71 ^a	3.8 (1.0–6.6), 7/183 ^a
Ansah et al., 2010	Ghana	Randomized controlled open label trial	98.3 (97.2–99.3), 565/575	49.5 (46.8–52.2), 656/1325
Bastiaens et al., 2011	Tanzania	Observational study	100.0, 12/12	82.5 (77.0–88.0), 151/183
D'Acremont et al., 2011	Tanzania	Cross-sectional surveys ^c	99, NR/370	53, NR/215
Masanja et al., 2012	Tanzania	Cross-sectional surveys	60.0 (51.9–68.1), 84/140 ^d	14.6 (10.2–19.1), 35/239 ^d
Yukich et al., 2012	Zambia	Cross-sectional study	66.1 ^f	NA

RDT, malaria rapid diagnostic test; BS, blood slide; NR, not reported; NA, not applicable.

^aCombined intervention and comparison arms, and blood slide and RDT-results pre-RDT and ^bpost-RDT implementation.

^cData derived from before-and-after analysis based on repeated cross-sectional surveys.

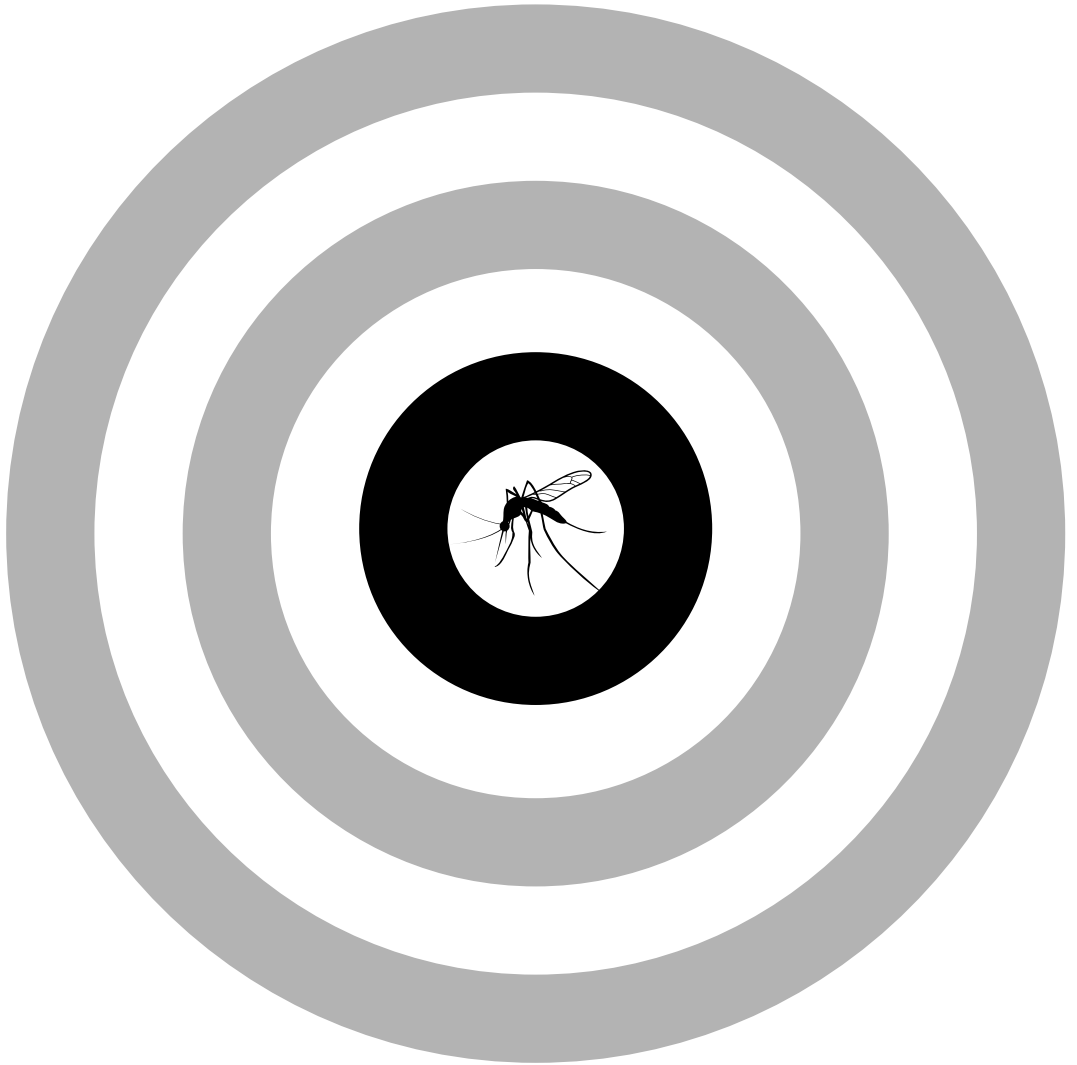
^dCombined blood slide and RDT-results pre-RDT implementation and ^epost-RDT implementation.

^fAntimalarial drug treatments per facility-month.

			Treatment with antibiotics	
	RDT		Microscopy	RDT
	% of RDT-positives	% of RDT-negatives	% of slide-negatives	% of RDT-negatives
	96.6 (93.6–99.5), 140/145	35.5 (28.6–42.5), 65/183	NR	NR
	98.9 (97.5–100.0), 186/188	53.7 (50.6–56.8), 540/1005	51.0 (47.9–54.0), 525/1030	51.6 (48.6–54.7), 519/1005
	56.7 (44.1–69.2), 34/60 ^b	6.2 (2.9–9.4), 13/210 ^b	NR	NR
	98.2 (97.1–99.2), 587/598	46.0 (43.3–48.7), 597/1298	28.6 (26.2–31.0), 379/1325	28.8 (26.3–31.3), 374/1298
	100.0, 26/26	16.6 (13.0–20.2), 67/404	62.8 (55.8–69.8), 115/183	92.8 (90.3–95.3), 375/404
	99, NR/126	7, NR/628	54, NR/215	78, NR/628
	71.8 (64.0–79.5), 94/131 ^e	7.0 (4.2–9.9), 22/313 ^e	NR	NR
	26.6 ^f	NA	NR	NR

SECTION B

MOSQUITOCIDAL ADDITIONS TO CURRENT ANTIMALARIAL TREATMENT TO PREVENT TRANSMISSION



CHAPTER 4

THE DURATION OF THE MOSQUITOCIDAL EFFECT OF IVERMECTIN

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Abstract

Introduction

Ivermectin (IVM) reduces the life span of malaria-transmitting mosquitoes after feeding on humans treated with IVM. If this effect is sufficiently long and strong, IVM could form part of a drug combination that not only treats malaria patients but also reduces onward transmission. Limited data are available on the exact duration of the mosquitocidal effect of IVM; daily mosquito feeding assays are required for this.

Methods

We determined mortality rates of *Anopheles stephensi* mosquitoes that took a blood meal on Swiss mice, Wistar rats and Cynomolgus monkeys that received IVM orally at 200–400 µg/kg. Mosquito feeding assays were performed on 5 consecutive days after IVM administration. Mosquito mortality was determined in the first 72 hours after feeding.

Results

Mosquito mortality was 70–100% when mosquitoes fed on any of the animals 1–2 days after the last IVM administration. After this time-point the mosquitocidal effect was still evident in most animals but it became more variable.

Discussion

Our findings of a pronounced but short-lived mosquitocidal effect makes the timing of IVM administration crucial to form a useful addition to antimalarial drugs.

Introduction

The search for malaria transmission-blocking drugs has so far focused on drugs that clear gametocytes, the sexual stage *Plasmodium* parasites that are responsible for the transmission of malaria to mosquitoes. An alternative or supplementary approach to prevent malaria transmission after treatment would be a strategy that aims to kill mosquitoes before they become infectious after feeding on a gametocytaemic blood meal. Ivermectin (IVM) may fulfil a role in such a strategy. IVM is a drug with broad-spectrum activity against nematodes and ectoparasites, and is widely used in mass treatment campaigns against onchocerciasis [1]. It has recently received much attention because of the reduced life span of malaria-transmitting mosquitoes after feeding on humans and cattle treated with IVM [2-5]. This makes IVM a potentially attractive component of malaria control efforts, where it could be part of a drug combination that not only treats malaria patients but also reduces onward transmission of the disease. The duration of the mosquitocidal effect of IVM is key to its potential role as an adjunct malaria therapy. Few data are available on the exact duration of this effect.

Fritz and colleagues showed previously that addition of IVM to bovine blood that is directly fed to *An. gambiae* sensu stricto and *An. arabiensis* mosquitoes reduces their survivorship and fecundity [5], and that IVM is already lethal to *An. arabiensis* at low concentrations (LC_{50} of 7.9 parts per billion) [6]. Work in rodents and cattle suggests a long-acting effect of IVM on anophelines, mosquito survival rates being affected as long as 10 days after feeding on an IVM-treated animal [5,7]. Chaccour and colleagues directly fed *An. gambiae* mosquitoes on healthy human volunteers 1 and 14 days post-IVM administration and observed an increased mortality at only the first time-point [2]. In a study from Papua New Guinea, blood-fed mosquitoes were collected from the huts of people 1–3 days and 28 days after an IVM mass treatment campaign. *An. punctulatus* or *An. koliensis* caught 1–3 days post-treatment showed 70% mortality in the first 24 hours after capture compared to 2% caught 28 days post-treatment [8]. A study from Senegal similarly determined survival in *An. gambiae* s.s. blood fed mosquitoes sampled from houses after IVM mass treatment campaigns and found that survival rates were lower for mosquitoes that were caught 1–6 days post-treatment; the effect for the individual days was not reported [4]. A study where *An. farauti* mosquitoes were allowed to feed on the skin of a single Indonesian volunteer 0, 7, 10, 14, 26 and 44 days after administration of 250 µg/kg IVM suggested that the mosquitocidal effect may last for 14 days [3].

In short, precise estimates of the duration of the mosquitocidal effect of IVM are unavailable and studies have been hampered by logistical and ethical challenges in performing frequently repeated mosquito feeding experiments. We aimed to support the discussion on the best strategy of IVM deployment by analyzing previously conducted and unpublished experiments on the mortality rate of *An. stephensi* mosquitoes after feeding on different animals treated with IVM at doses within the range that is recommended for use in humans (200–400 µg/kg).

Methods

Experiments were performed at the Radboud University Nijmegen Medical Centre animal facility in the period May 1986–October 1987, where the test animals and mosquitoes were housed according to local and national guidelines. Permission to conduct the experiments in this study was given under approval number DGVGZ/VVP-83262.

Swiss mice (*Mus musculus*; $n=10$) weighing ~25 g each were given forage containing 2 mg/kg IVM. Oral take-up of 5 g of forage per day resulted in a total take-up of 10 μg IVM per day per mouse, or a daily dose of 400 $\mu\text{g}/\text{kg}$ IVM. Four to 5 days old *An. stephensi* mosquitoes were allowed to feed directly on the skin of all animals exactly 1, 2, 3, 4 and 5 days after IVM treatment. After each feeding experiment fully engorged mosquitoes were selected. The median number of fully engorged mosquitoes per mouse per feeding day was 10 (interquartile range [IQR], 10–13.25; total number of mosquitoes, 503). Mosquito mortality for each of the 5 mosquito feeding days was compared with 15 mosquito feeding experiments on untreated Swiss mice; an entirely separate group of animals. Mice were given IVM treatment for 1–5 days prior to the first feeding experiment. The total dose of IVM therefore differed between animals. However, we observed no effect of longer treatment on mosquito mortality after the last dose of IVM (Table 1; $P=0.87$) and animals were therefore combined and analysed as a group.

Wistar rats (*Rattus norvegicus*, $n=5$) weighing ~120 g each were given Ivomec® solution orally. Ivomec® solution containing 10 $\mu\text{g}/\text{mL}$ IVM was diluted to 100 $\mu\text{g}/\text{mL}$ IVM using distilled water, and administered orally by syringe to reach a daily dose of 400 $\mu\text{g}/\text{kg}$ IVM. Mosquito feeding assays were performed as above. The median number of fully engorged mosquitoes per rat per feeding day was 26 (IQR, 20–30; total number of mosquitoes, 673). Mosquito mortality for each of the five mosquito feeding days was compared with 15 mosquito feeding experiments on a completely separate group of untreated Wistar rats. While IVM was given 1–5 days prior to mosquito feeding, we observed no effect of longer treatment on mosquito mortality after the last dose of IVM ($P=0.83$) and animals were therefore combined and analysed as a group.

Cynomolgus monkeys (*Macaca fascicularis*, $n=3$) were given an orange containing Ivomec® solution at a single dose of 200 ($n=2$) or 400 $\mu\text{g}/\text{kg}$ bodyweight ($n=1$). Mosquito feeding assays were performed as above. The median number of fully engorged mosquitoes per monkey per feeding day was 14 (IQR, 10.5–16.5) and was not recorded for all experiments. For some experiments only the proportion of surviving mosquitoes was recorded; this did not affect the statistical analysis where we used a single estimate per mosquito feeding experiment (see below) but limited the information that could be given for individual experiments on Cynomolgus monkeys (Table 1). Mosquito mortality for each of the 5 mosquito feeding days was compared with 21 mosquito feeding experiments on a separate group of untreated Cynomolgus monkeys.

Table 1 Mosquito feeding experiments after ivermectin treatment in different animals

Animal species	Number of animals	Number of treatment days	Daily dose of IVM	Mortality experiment day 1* %(n/N)	Mortality experiment day 2* %(n/N)	Mortality experiment day 3* %(n/N)	Mortality experiment day 4* %(n/N)	Mortality experiment day 5* %(n/N)
Swiss mouse	2	1	400 µg/kg	100 (17/17)	89.5 (17/19)	0.0 (0/7)	0.0 (0/11)	9.1 (1/11)
	2	2	400 µg/kg	100 (17/17)	95.2 (20/21)	85.7 (18/21)	37.5 (6/16)	65.6 (21/32)
	2	3	400 µg/kg	100 (22/22)	87.5 (21/24)	0.0 (0/6)	6.3 (1/16)	0.0 (0/16)
	2	4	400 µg/kg	100 (20/20)	100 (24/24)	66.7 (18/27)	50.0 (11/22)	30.4 (7/23)
	2	5	400 µg/kg	100 (25/25)	88.5 (23/26)	67.9 (19/28)	52.0 (13/25)	0.0 (0/27)
Wistar rat	1	1	400 µg/kg	100 (22/22)	100 (24/24)	65.4 (17/26)	44.1 (15/34)	29.4 (10/34)
	1	2	400 µg/kg	92.9 (26/28)	85.0 (17/20)	96.2 (25/26)	42.9 (15/35)	16.7 (3/18)
	1	3	400 µg/kg	85.0 (17/20)	100 (29/29)	72.4 (21/29)	53.8 (21/39)	45.7 (16/35)
	1	4	400 µg/kg	84.6 (22/26)	91.7 (22/24)	66.7 (12/18)	13.3 (4/30)	18.5 (5/27)
Cynomolgus monkey	1	5	400 µg/kg	100 (16/16)	100 (19/19)	77.8 (14/18)	31.4 (11/35)	35.7 (10/28)
	1	1	200 µg/kg	100 (10/10)	76.9 (10/13)	37.5 (3/8)	14.3 (1/7)	12.5 (1/8)
	1	1	200 µg/kg	100**	91**	86**	0**	0**
	1	1	400 µg/kg	100**	92**	45**	72**	8**

*determined in the first 72 hours after feeding; **only the proportion of dead mosquitoes was recorded for these experiments; IVM, ivermectin.

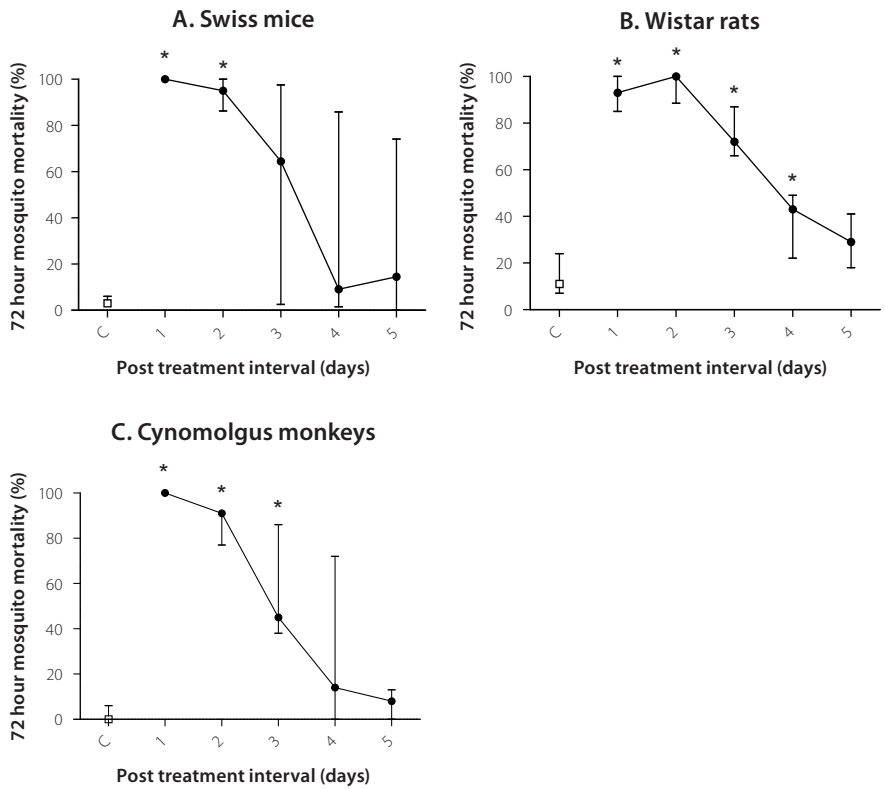


Figure 1 Mosquito mortality within 72 hours after feeding on different animals treated with ivermectin

Mosquito feeding experiments were conducted on 5 consecutive days following IVM treatment. Mosquito mortality was calculated for the first 72 hours after each mosquito feeding experiment. Plotted on the x-axis is the day since the last IVM dose, plotted on the y-axis is the proportion of all fully fed mosquitoes that died within 72 hours after their blood meal. Swiss mice (n=10) and Wistar rats (n=5) received IVM 400 µg/kg for 1–5 days (A and B). Cynomolgus monkeys (n=3) received a single dose of IVM at 200 (n=2) or 400 µg/kg (n=1) (C). Dots indicate the median mosquito mortality and error bars indicate the interquartile range; for IVM-treated monkeys this interquartile range equals the total range because only 3 monkeys received IVM. Lines are drawn between the estimates of the median and do not represent fitted or smoothed curves.

*statistically significant after adjusting p-values for multiple comparisons.

For each of the 5 time-points after IVM, the proportion of mosquitoes that died within 72 hours after their blood meal was compared with control experiments using STATA version 12.0 (StataCorp LP, Texas, US). Because the number of animals was deemed too small to reliably estimate clustering of individual mosquito observations that fed on the same animal, we chose the most conservative analytical approach: each feeding experiment contributed one observation of the proportion of mosquitoes that died within 72 hours after this feeding experiment. This continuous variable was compared between test and control experiments using a nonparametric Wilcoxon rank-sum test; a Bonferroni correction was used to counteract the problem of multiple comparisons.

Results

In mice, the proportion of mosquitoes that died within 72 hours after their blood meal was strongly elevated when feeding 1 or 2 days after IVM (Table 1; Figure 1a). On these days 70–100% of mosquitoes died within 72 hours after feeding on any of the IVM-treated mice compared to 0–10% in control mice ($P<0.0001$ for both days). Beyond day 2, higher mosquito mortality was still observed in some animals but we no longer found evidence for a statistically significantly elevated mosquito mortality compared to control mice. For rats, mosquito mortality was also most pronounced for mosquitoes feeding one or two days after IVM: 85–100% of all mosquitoes died within 72 hours after feeding on any of the IVM-treated rats compared to 2–28% in control rats (Table 1; Figure 1b; $P=0.001$ for day 1 and $P=0.0009$ for day 2). Mosquito mortality decreased after this time-point but remained statistically significantly elevated up to day 4 after IVM treatment ($P=0.008$). Similar as in rats and mice, mosquito mortality in experiments with *Cynomolgus* monkeys was highest when feeding one or two days after IVM (Table 1; Figure 1c; 77–100% mosquito mortality within 72 hours after feeding, $P=0.002$ for both days). On the third day after treatment, mosquito mortality was more variable (38–86%) although still statistically significantly higher than control experiments ($P=0.002$).

Discussion

We observed a pronounced effect of IVM on mosquito mortality rates in all animal models. The strong but short-lived mosquitocidal effect of IVM that we observed supports several previous observations [2,4,8] with more detailed daily assessments and a larger number of mosquito observations.

A strength of our study is that we have performed daily mosquito feeding experiments in 3 animal species and thereby are able to add an estimate of mortality on different days post-IVM to the available literature. Since the mosquitocidal effect of IVM was previously shown to be highly dependent on the concentration of IVM in plasma [9], we interpret our finding that the mosquitocidal effect becomes more variable after 2 days, as an indication

for inter-individual variation in IVM metabolism. Since we did not determine IVM plasma concentrations in our study animals, we were unable to address this further. This is a shortcoming of the current study. We found no evidence in literature that the plasma disposition of IVM differs between our animal models and humans. Since our findings are consistent between the different animals, we consider it plausible that also in humans the mosquitocidal effect of IVM is limited to the first days after treatment.

Our findings have implications for the next steps in evaluating IVM as a tool for use in malaria control. The strong but short-lived mosquitocidal effect makes IVM less attractive as a stand-alone drug for mass drug administration (MDA) campaigns that aim to reduce malaria transmission. IVM may, however, be a potent addition to antimalarial drugs in MDA campaigns or to prevent transmission shortly after treatment of symptomatic malaria cases. Current therapy for malaria patients is based on treatment with artemisinin combination therapy (ACT). Whilst ACTs are highly effective against asexual parasite stages and immature gametocytes, mature gametocytes persist for several weeks after treatment [10]. As a result, there is a pronounced but incomplete reduction of malaria transmission in the first 7–14 days after treatment [11–13]. Primaquine (PQ) is the only currently available drug that can play a role in reducing the infectious period after ACT by actively clearing mature gametocytes. Addition of PQ to ACT reduces the duration of gametocyte carriage 4-fold compared to ACTs alone [14]. However, a single dose of PQ at the currently recommended concentration (0.75 mg/kg) is associated with haemolysis in glucose-6-phosphate-dehydrogenase (G6PD) deficient individuals [15,16]. This sub-optimal safety profile hinders wide-scale use of PQ in malaria control [17]. There are currently no safe alternatives to PQ available, although dose-finding studies to determine a lower but efficacious dose of PQ are ongoing and the gametocytocidal activity and safety profile of the drug candidate methylene blue are promising [18].

Adding IVM to ACTs may be a promising strategy to further reduce post-treatment malaria transmission. For this, the timing of IVM administration has to be optimized to cover the period when an individual is most infectious, which is plausibly the first week after initiation of treatment with ACTs [11,19]. An attractive element of IVM as a ‘targeted vector control tool’ is that it acts against both indoor and outdoor biting vectors and targets different effector molecules compared to indoor residual spraying with insecticides and insecticide treated nets [20,21]. An additional benefit in terms of integrated disease management would be the curative effect of IVM on intestinal strongyloidiasis, onchocerciasis and scabies [1]. Several important safety and tolerability data of an ACT-IVM drug combination are needed to confirm its potential, as well as pharmacokinetic data to ensure there are no drug interactions that may reduce the efficacy of the ACT or partner drug component.

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CHAPTER 5

EFFICACY AND SAFETY OF THE MOSQUITOCIDAL DRUG IVERMECTIN TO PREVENT MALARIA TRANSMISSION AFTER TREATMENT: A DOUBLE-BLIND, RANDOMIZED, CLINICAL TRIAL

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Abstract

Introduction

Artemisinin combination therapy effectively clears asexual malaria parasites and immature gametocytes but does not prevent post-treatment malaria transmission. Ivermectin (IVM) may reduce malaria transmission by killing mosquitoes that take blood meals from IVM treated humans.

Methods

In this double-blind, placebo-controlled trial, 120 asymptomatic *Plasmodium falciparum* parasite carriers were randomized to receive artemether-lumefantrine (AL) plus placebo or AL plus a single or repeated dose (200 µg/kg) of ivermectin (AL-IVM1 and AL-IVM2, respectively). Mosquito membrane feeding was performed 1, 3, and 7 days after initiation of treatment to determine *Anopheles gambiae* and *Anopheles funestus* survival and infection rates.

Results

The AL-IVM combination was well tolerated. IVM resulted in a 4- to 7-fold increased mortality in mosquitoes feeding 1 day after IVM ($P<0.001$). Day 7 IVM plasma levels were positively associated with body mass index ($r=0.57$, $P<0.001$) and were higher in female participants ($P=0.003$), for whom *An. gambiae* mosquito mortality was increased until 7 days after a single dose of IVM (hazard rate ratio, 1.34 [95% confidence interval, 1.07–1.69]; $P=0.012$). Although we found no evidence that IVM reduced *Plasmodium* infection rates among surviving mosquitoes, the mosquitocidal effect of AL-IVM1 and AL-IVM2 resulted in 27% and 35% reductions, respectively, in estimated malaria transmission potential during the first week after initiation of treatment.

Discussion

We conclude that IVM can be safely given in combination with AL and can reduce the likelihood of malaria transmission by reducing the life span of feeding mosquitoes.

Introduction

The transmission of *Plasmodium* from humans to mosquitoes depends on the presence of mature transmission stages, gametocytes. Once ingested, gametocytes may render mosquitoes infectious within 11–16 days after a blood meal [1]. Artemisinin combination therapy (ACT) forms the current first-line treatment for uncomplicated falciparum malaria. ACT rapidly clears asexual parasites and developing gametocytes but leaves mature *P. falciparum* gametocytes largely unaffected; a proportion of patients may transmit malaria after successful ACT treatment [2]. Strategies to prevent malaria transmission after ACT have received a sense of urgency with the emergence of artemisinin-resistance in Southeast Asia [3,4] and have mainly focused on supplementing ACT with gametocytocidal compounds [5–7]. An alternative approach to prevent post-treatment malaria transmission is to reduce the likelihood that mosquitoes that feed on gametocytaemic human hosts survive long enough to become infectious to other humans. Ivermectin (IVM) reduces the lifespan of *Anopheles* mosquitoes that feed on humans who have taken IVM [8,9] by activating glutamate-gated chloride (GluCl) channels in neuronal and neuromuscular tissues of invertebrates, thereby causing flaccid muscle paralysis [10]. IVM has an excellent safety profile in humans, allowing IVM to be used in mass drug administration campaigns to reduce the burden of onchocerciasis and lymphatic filariasis in Africa. IVM has never been tested in a clinical trial setting in malaria-infected individuals or in combination with ACT.

In this study, we report a randomized, double-blind, placebo-controlled clinical trial to determine the safety and impact of IVM, administered as single or repeated dose, in combination with artemether-lumefantrine (AL) in reducing the proportion of mosquitoes that survive sufficiently long to complete the sporogonic cycle of malaria.

Methods

Study design and participants

This trial was conducted from January until March 2013 in Balonghin, Burkina Faso. Individuals aged 15–25 were eligible if found to be infected with *P. falciparum* by microscopy and otherwise healthy. Exclusion criteria were: $\geq 20,000$ malaria parasites/ μL ; severe malaria; fever (axillary temperature $\geq 37.5^\circ\text{C}$); haematological or biochemical abnormalities; body mass index (BMI) < 16 or > 32 kg/m^2 ; haemoglobin concentration < 11 g/dL ; use of IVM within the previous 3 months; *Loa loa* or other filariasis infection; travel history to *L. Loa*-endemic areas; pregnancy or lactation; current tuberculosis or antiretroviral treatment; family history of congenital QTc interval prolongation or sudden death; use of drugs that influence cardiac function or prolong QTc interval; or electrolyte imbalance. Written informed consent was obtained. The trial was approved by the Interventions Research Ethics Committee of the London School of Hygiene and Tropical Medicine (reference number 6154), Comité d’Ethique pour la Recherche en Santé, Ministère de la Santé du

Burkina Faso (reference number 2012-5-026) and Comité Technique d'Examen des Demandes d'Autorisation d'Essais Cliniques, Ministère de la Santé du Burkina Faso (reference number 50001020125EC00000).

Randomization and masking

Included subjects (n=120) were randomly assigned to 1 of 3 treatment arms and 1 of 2 membrane feeding schedules. A first set of 40 sealed envelopes contained cards indicating treatment with AL alone (AL, n=20) or AL with a single dose of IVM (AL-IVM1, n=20). After reviewing safety data, 80 additional participants were randomized to AL (n=20), AL-IVM1 (n=20), or AL with a repeated treatment dose of IVM (AL-IVM2, n=40). Half of each treatment arm was allocated to membrane feeding on days 1 and 7, others to days 3 and 7.

Procedures

All subjects received 6 doses of 4 tablets of AL (Coartem [20 mg artemether and 120 mg lumefantrine], Novartis Pharma AG, Basel, Switzerland) at enrolment and after 8 hours (day 0), 24 and 36 hours (day 1), and 48 and 60 hours (day 2) (± 90 minutes). In the AL arm, the first and fifth dose of AL were given together with placebo tablets (Albochin, Pharmachemie BV, Haarlem, The Netherlands); in the AL-IVM1 arm, the first dose of AL was given with IVM (Stromectol, Merck Sharp & Dohme BV, Haarlem, The Netherlands) and the fifth AL dose together with placebo. In the AL-IVM2 arm, both the first and fifth AL dose were given together with IVM. IVM was given as 3-mg tablets aiming for a dose of 200 $\mu\text{g}/\text{kg}$. All treatment was administered under direct supervision, with 1 sachet of Nestle NIDO powder milk (containing 7.28 g of milkfat) dissolved in water to enhance bio-availability of AL [11].

Participants were examined clinically on days 1, 2, 3, and 7 after enrolment. Blood samples were taken for microscopy (days 0, 3, and 7), standard haematological and biochemical parameters (days 0 and 7), membrane feeding assays (days 1 and 7 or days 3 and 7), pharmacological assessment (days of membrane feeds) and gametocyte detection by Pfs25 messenger RNA quantitative nucleic acid sequence-based amplification (QT-NASBA; days 0, 3, and 7) [7].

Membrane feeding assays were conducted as described elsewhere [12] using 100–150 locally reared 4 to 5-day-old female *Anopheles gambiae* sensu stricto mosquitoes and 50–70 4 to 5-day-old *Anopheles funestus* mosquitoes. Because of mosquito husbandry limitations, experiments with *An. funestus* were done with a smaller number of mosquitoes and on days 1 and 3 only. Fully fed mosquitoes were kept on glucose for 10 days at 27°C–29°C to monitor daily mosquito mortality. *Anopheles gambiae* mosquitoes that survived until day 10, when residual DNA from the blood meal is highly unlikely [13,14], were individually homogenised and processed for detection of *P. falciparum* oocysts or sporozoites by polymerase chain reaction (PCR) [14]. On day 7, lumefantrine plasma concentrations were determined for 20 randomly selected individuals per treatment arm

[15]; on days 1, 3, and 7, IVM plasma concentrations were determined for all individuals participating in membrane feeding experiments using high-performance liquid chromatography with fluorescence detection and a sensitivity of 0.2 ng IVM/mL [16].

Outcome measures

The study objective was to determine the safety and efficacy of IVM in combination with AL in reducing the proportion of mosquitoes that survive long enough to complete the sporogonic cycle of *P. falciparum*. The primary efficacy endpoint was the survival of *An. gambiae* and *An. funestus* mosquitoes after taking a blood meal 1, 3 or 7 days after initiation of treatment. Plasma concentrations of AL and IVM after treatment and mosquito infection rates were secondary outcome measures. The associations of IVM plasma concentrations with sex and BMI were not initially defined in the study protocol.

Statistical analysis

For the primary efficacy outcome, individual mosquito data were analysed by Cox proportional hazard models with shared frailty to allow for the correlation between mosquito observations from the same donor. Cumulative mosquito mortality by day 10 after feeding was determined for each individual membrane feeding experiment, \log_{10} -transformed, and compared with the AL reference arm using *t* test. IVM (days 1, 3, 7) and lumefantrine plasma concentrations (day 7) were compared between groups using nonparametric Wilcoxon-rank sum test. Proportions were compared between arms by χ^2 test, associations between continuous variables were determined by Spearman correlation coefficients, and the association between sex and IVM plasma concentrations was determined by Wilcoxon rank-sum test.

The impact of IVM on transmission from patients during the first week after initiation of treatment was estimated using data from a clinical trial with detailed gametocyte quantification after AL [7], a meta-analysis of the association between gametocyte concentration and *An. gambiae* mosquito infection rates [17], and *An. gambiae* mosquito survivorship in relation to IVM concentration. Assuming that a similar number of mosquitoes would feed on individuals from treatment arms and on all days of follow-up, the impact of IVM at reducing the number of infectious mosquitoes can be calculated as

$$\frac{\sum_{i=0}^7 (g_i p_i \mu_i^{AL}) - \sum_{i=0}^7 (g_i p_i \mu_i^{IVM})}{\sum_{i=0}^7 (g_i p_i \mu_i^{AL})}$$

where g_i is the gametocyte prevalence at each day of follow-up (i) after treatment, p_i is the proportion of mosquito infection in feeding assays with gametocyte levels at day i , and μ_i is the proportion of mosquito survival up to day 10.

Sample size calculation

This study was designed as a superiority trial, testing mosquito mortality in the 2 IVM arms against the AL comparator arm. The study sample size was based on 80–100 fully fed *An. gambiae* sensu stricto mosquitoes and $\leq 20\%$ mortality in the control arm [12]. Including 20 individuals per time-point would allow us to detect an increase in mortality to $\geq 50\%$ after 1 or 2 doses of IVM compared to the control arm ($k=0.5$; $Z_{\alpha}/2=1.96$; $Z_{\beta}=0.84$). For day 7, we expected the smallest difference in mortality and aimed for 40 experiments per treatment arm.

Results

Trial profile and baseline characteristics

Of 120 randomized individuals, 117 completed follow-up (Figure 1). Baseline asexual parasite densities ranged from 8 to 7063 parasites/ μL (Table 1); all participants cleared their asexual parasites by day 3. Gametocyte prevalence by QT-NASBA declined from 91.9% (102/111) at baseline to 54.9% (62/113) by day 3 and 41.8% (43/103) by day 7 with no significant difference between treatment arms ($P \geq 0.81$).

Safety results

Twenty-two adverse events (AEs) occurred; 10 AEs were ranked as mild and 12 as moderate in intensity (Table 2). None of the AEs were definitively associated with treatment and no serious AEs were seen. Platelet counts declined in 2 subjects during follow-up. In 1 subject of the AL group, platelets decreased from 327,000/ μL at enrolment to 82,800/ μL on day 7. This subject refused to return to the clinic for extra follow-up and was followed passively. In 1 subject of the AL-IVM1 group, platelets decreased from 191,000/ μL at enrolment to 68,500/ μL on day 7, and returned to 218,000/ μL when measured 20 days later. There were no other clinically significant haematological and biochemical abnormalities.

Efficacy results

The median number of fully fed *An. gambiae* mosquitoes was 94 per experiment (interquartile range [IQR], 92–96) and not different between treatment arms ($P=0.15$), giving 22,818 mosquito observations from 233 experiments conducted on days 1, 3, and 7 post-treatment. The median number of fully fed *An. funestus* mosquitoes was 23 per experiment (IQR, 23–25) and not different between treatment arms ($P=0.19$), giving 2,469 mosquito observations from 102 experiments conducted on days 1 and 3. *Anopheles gambiae* mortality was significantly increased on day 1 after single-dose IVM (hazard rate ratio [HRR], 3.86 [95% confidence interval {CI}, 3.29–4.52]; $P<0.001$), day 3 after single-dose IVM (HRR, 1.37 [95% CI, 1.14–1.65]; $P=0.001$), day 3 after repeated-dose IVM (HRR, 4.07 [95% CI, 3.41–4.87]; $P<0.001$), and day 7 after repeated-dose IVM (HRR, 1.30 [95% CI, 1.10–1.53]; $P=0.002$), but not day 7 after single-dose IVM (HRR, 0.93 [95% CI, 0.79–1.11]; $P=0.43$).

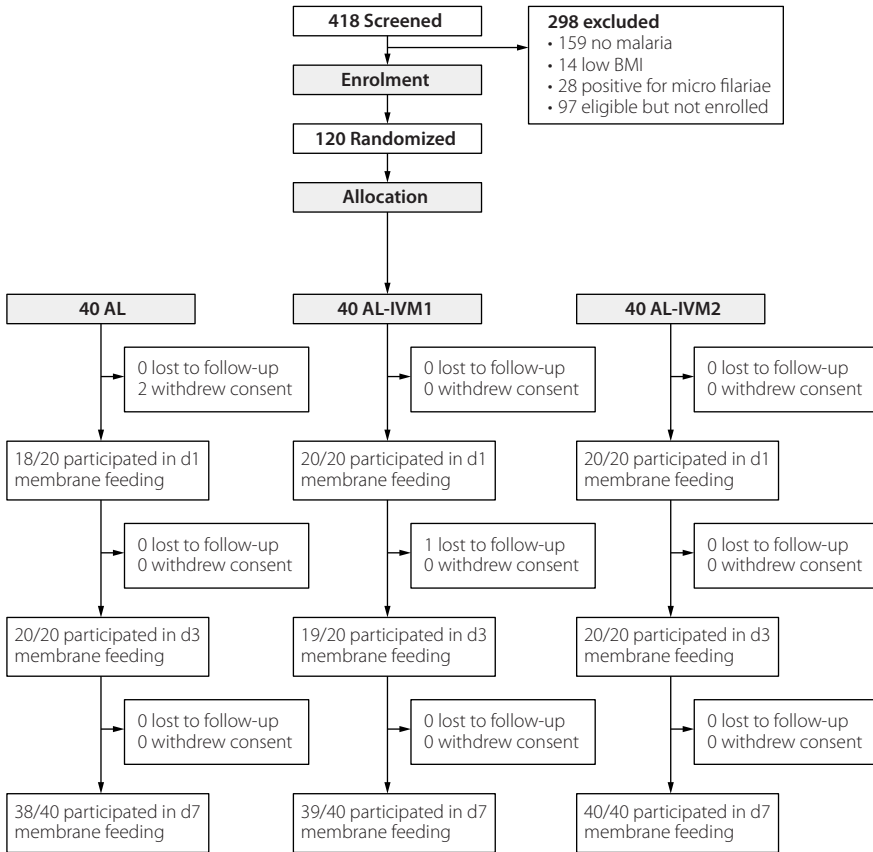


Figure 1 Trial profile

Membrane feeding participation rates are reported for *Anopheles gambiae*.

AL, artemether-lumefantrine; BMI, body mass index; IVM1, single-dose ivermectin; IVM2, repeated-dose ivermectin.

(Figure 2A). Similarly, *An. funestus* mosquito mortality was significantly increased on day 1 after single-dose IVM (HRR, 7.12 [95% CI, 4.45–11.39]; $P<0.001$), day 3 after single-dose IVM (HRR, 2.98 [95% CI, 1.62–5.48]; $P<0.001$), and day 3 after repeated dose IVM (HRR, 9.07 [95% CI, 5.06–16.25]; $P<0.001$) (Figure 2B). Geometric mean cumulative *An. gambiae* mosquito mortality by day 10 after membrane feeding was 21.2% (95% CI, 18.5%–24.3%) in the AL arm; 59.1% (95% CI, 53.3%–65.6%; $P<0.001$) on day 1 after single-dose IVM, 31.1% (95% CI, 26.5%–36.5%; $P=0.001$) on day 3 after single-dose IVM; 66.2% (95% CI, 58.5%–74.9%; $P<0.001$) on day 3 after repeated-dose IVM; 21.7% (95% CI, 18.5%–25.4%, $P=0.82$) on day 7

Table 1 Baseline characteristics of enrolled subjects

Characteristic	Treatment Group		
	AL (n=40)	AL-IVM1 (n=40)	AL-IVM2 (n=40)
Age, y, median (IQR)	18.5 (16.0–21.3)	16.0 (15.0–20.0)	17.0 (16.0–18.5)
Sex, male, % (n/N)	65.0% (26/40)	45.0% (18/40)	70.0% (28/40)
Haemoglobin, g/dL, median (IQR)	13.3 (12.0–14.4)	13.2 (12.3–13.9)	12.9 (12.3–13.8)
Parasitaemia by microscopy, parasites/ μ L, median (IQR)	109.5 (38.3–222.0)	87.0 (28.0–203.5)	134.0 (45.0–406.0)
Gametocyte prevalence by microscopy, % (n/N)	20.0 (8/40)	12.5 (5/40)	12.5 (5/40)
Gametocyte prevalence by QT-NASBA, % (n/N)	97.2% (35/36)	86.8% (33/38)	91.9% (34/37)

AL, artemether-lumefantrine; IQR, interquartile range; IVM1, single-dose ivermectin; IVM2, repeated-dose ivermectin; QT-NASBA, quantitative nucleic acid sequence-based amplification.

Table 2 Adverse events of any severity in the different treatment arms

Adverse Event	Treatment Group		
	AL (n=38)	AL-IVM1 (n=39)	AL-IVM2 (n=40)
Abdominal pain		2	
Abscess on hand			1
Abscess on leg			1
Bronchitis	1		
Conjunctivitis		1	
Cough		1	1
Dental pain			1
Diarrhoea		1	
Fever	1		
Headache	1	1	4
Orchitis		2	
Painful swelling of leg			1
Pharyngitis			1
Urinary tract infection			1

AL, artemether-lumefantrine; IVM1, single-dose ivermectin; IVM2, repeated-dose ivermectin.

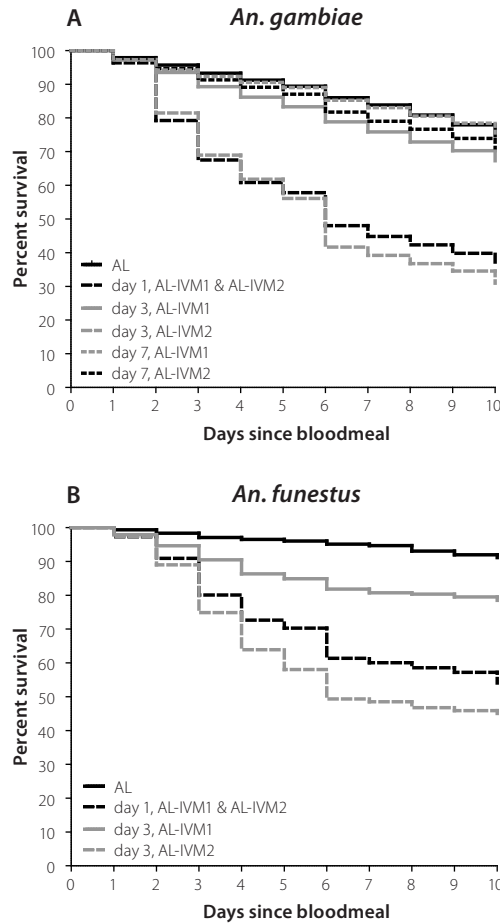


Figure 2 Mosquito survival rate in relation to treatment arm for *Anopheles gambiae* and *Anopheles funestus*

The proportion of *An. gambiae* (A) or *An. funestus* (B) that survive in 10 days following membrane feeding in various treatment arms at days 1, 3, and 7 was combined for artemether-lumefantrine (AL); day 1 was combined for AL with single-dose ivermectin (IVM1) and AL with repeated-dose ivermectin (IVM2) as this was before the second dose of ivermectin.

after single-dose IVM; and 26.7% (95% CI, 23.2%–30.7%; $P=0.013$) on day 7 after repeated-dose IVM. Geometric mean cumulative *An. funestus* mosquito mortality was 5.0% (95% CI, 3.2%–7.8%) in the AL arm; 40.0% (95% CI, 26.8%–59.8%; $P<0.001$) on day 1 after single-dose IVM; 10.9% (95% CI, 5.4%–22.0%; $P=0.033$) on day 3 after single-dose IVM, and 51.4% (95% CI, 37.7%–69.9%; $P<0.001$) on day 3 after repeated-dose IVM.

Median lumefantrine concentration was 685 ng/mL (IQR, 474–894 ng/mL) in the AL arm, 634 ng/mL (IQR, 420–818 ng/mL) in the AL-IVM1 arm, and 449 ng/mL (IQR, 385–734 ng/mL) in the AL-IVM2 arm ($P=0.28$). IVM plasma concentrations declined quickly after the last dose of IVM (Figure 3a) and were significantly higher in female than in male participants when measured on day 3 after single-dose IVM ($P=0.02$), and day 7 after single-dose ($P=0.007$) or repeated-dose IVM ($P=0.003$). IVM accumulates in fat tissue [18] and the proportion of body fat is positively associated with BMI. BMI was associated with IVM plasma concentration on day 3 (IVM1: $n=18$, $r=0.64$, $P=0.004$; IVM2: $n=20$, $r=0.19$, $P=0.42$), and day 7 (IVM1: $n=37$, $r=0.73$, $P<0.0001$; IVM2: $n=40$, $r=0.49$, $P=0.001$; Figure 3b) but not on day 1. Female participants had a higher mean BMI than male participants (difference of means, 1.12 kg/m^2 [95% CI, $0.54\text{--}1.71 \text{ kg/m}^2$]; $P=0.0002$). IVM plasma concentrations were strongly associated with cumulative mortality by day 10 after the blood meal for *An. gambiae* ($r=0.75$, $P<0.0001$; Figure 4) and *An. funestus* ($r=0.48$; $P<0.0001$). When stratified by sex, the lethal effect of IVM on *An. gambiae* was more pronounced and longer in women (Table 3). The number of *An. funestus* observations was 9-fold lower than for *An. gambiae* and considered too limited to allow robust sex-stratified analysis.

Table 3 Hazard rate ratios for *Anopheles gambiae* mortality on different days after initiation of treatment

	Male participants		Female participants	
Treatment	Hazard rate ratio	P-value	Hazard rate ratio	P-value
No ivermectin	1 (ref)		1 (ref)	
Day 1, IVM1 & IVM2	3.29 (2.67–4.07)	<0.001	5.03 (4.00–6.31)	<0.001
Day 3, IVM1	1.39 (1.04–1.85)	0.025	1.67 (1.31–2.13)	<0.001
Day 3, IVM2	3.54 (2.81–4.46)	<0.001	5.23 (4.01–6.83)	<0.001
Day 7, IVM1	0.70 (0.55–0.89)	0.003	1.34 (1.07–1.69)	0.012
Day 7, IVM2	1.14 (0.92–1.42)	0.23	1.61 (1.25–2.08)	<0.001

Hazard rate ratios were determined relative to the artemether-lumefantrine placebo arm and adjusted for the correlation between observations from the same individual.

IVM1, single-dose ivermectin; IVM2, repeated-dose ivermectin.

Individual *An. gambiae* mosquitoes from 68 membrane feeds performed on days 1 and 7 on Pfs25 QT-NASBA-confirmed gametocyte carriers were successfully analysed by PCR. Remaining assays failed because of freeze-thaws of mosquito samples, giving non-interpretable results. In total, only 0.8% (13/1,619) of the successfully assayed mosquitoes were *P. falciparum* positive: 0.7% (4/560) in the AL-arm, 0.5% (3/556) in the AL-IVM1 arm, and

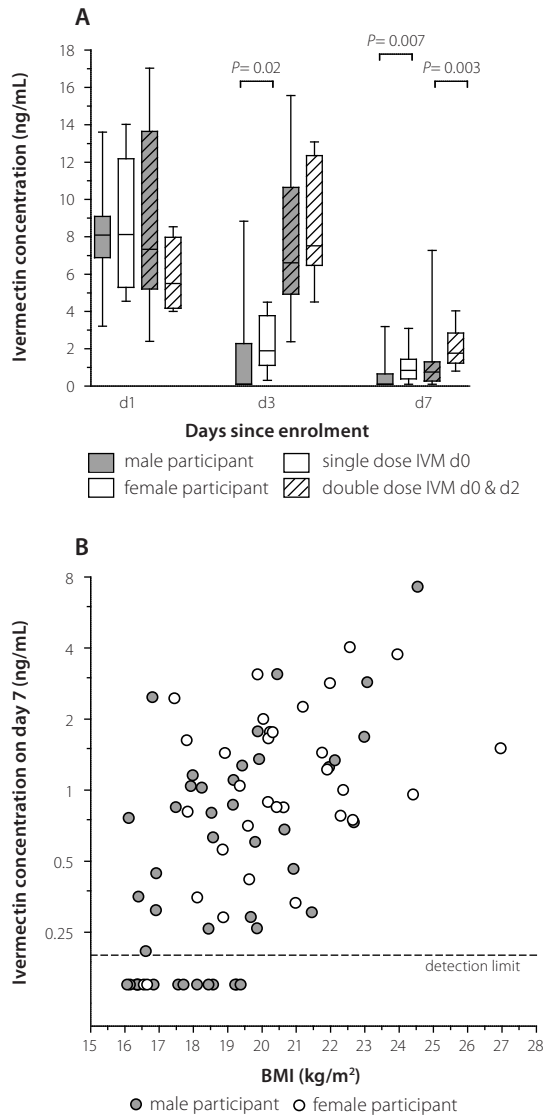


Figure 3 Ivermectin plasma concentrations in relation to the sex of participants and body mass index

(A) Sex-stratified IVm plasma concentration for the different treatment arms and time-points of follow-up. **(B)** Association between BMI and day 7 IVm plasma concentrations for male (gray dots; $n=45$; $r=0.52$, $P=0.0002$) and female ($n=32$; $r=0.37$, $P=0.037$) participants. The limit of detection of the assay was 0.2 ng/mL (dashed line); plasma samples with undetectable IVm concentrations are given below the line.

1.2% (6/503) in the AL-IVM2 arm. Supporting in vitro experiments found no apparent effect of sublethal IVM concentrations on *P. falciparum* development in *An. stephensi* and *An. gambiae* mosquitoes (Supporting Information 1).

We combined our longitudinal data on IVM concentrations (Figure 3), our data on the association between IVM concentration and *An. gambiae* mosquito survivorship (Figure 4), previously published data on gametocyte prevalence and density following treatment of symptomatic malaria patients [7], and a meta-analysis of the association between gametocyte density and mosquito infection rates [17] to estimate the potential impact of IVM on onward malaria transmission in the first week after initiation of treatment (Supporting Information 2). Despite the incomplete and short-lived mosquitocidal effect of IVM, mosquito survivorship is significantly reduced in the first days after treatment when gametocyte concentrations are highest and onward transmission is most likely. Compared to the AL-only arm, we estimated that individuals in the AL-IVM1 and AL-IVM2 arms had a 27.2% and 35.4% reduction, respectively, in their contribution to transmission in the first week after initiation of treatment (Figure 5).

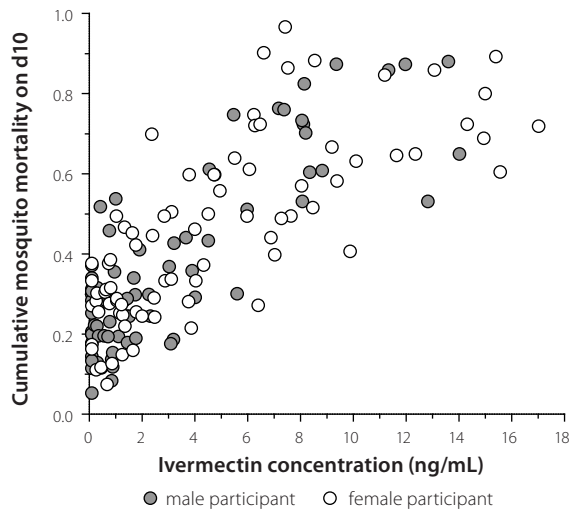


Figure 4 Ivermectin plasma concentrations in relation to cumulative mortality of *Anopheles gambiae*

The association between IVM plasma concentrations (all time-points combined) and cumulative *An. gambiae* mosquito mortality by day 10 (d10) after taking a blood meal. $r=0.75$, $P<0.0001$.

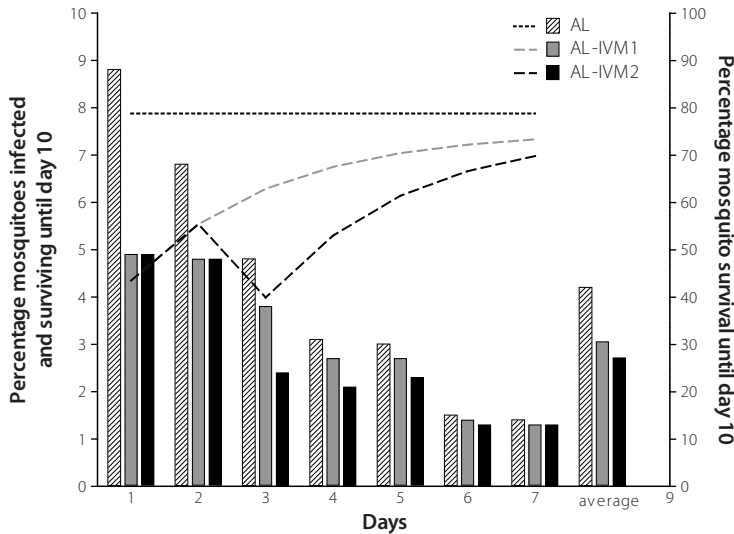


Figure 5 The estimated impact of treatment with ivermectin on *Plasmodium falciparum* transmission for malaria patients during the first week after treatment with artemether-lumefantrine

Bars indicate the percentage of mosquitoes that become infected and survive for 10 days after their blood meal for the different treatment arms and days after treatment (left y-axis). The assumptions underlying these estimates are outlined in the Supporting Information and are based on gametocyte prevalence and density estimates from Ugandan children aged 1–10 years [7], a meta-analysis of mosquito feeding experiments [17], and IVM pharmacokinetics and mosquito survival rates presented in the current manuscript. Lines indicate mosquito survival rates for the different treatment arms (right y-axis) and were either directly estimated or based on the best fit of IVM pharmacokinetic data (Figure 2a) and the association between IVM plasma concentration and mosquito infection rates (Figure 3). Mosquito mortality in the AL-only arm (21% over 10 days) is considered to reflect natural mortality over this period and is assumed to be unrelated to the ingestion of gametocytes.

IVM1, single-dose ivermectin; IVM2, repeated-dose ivermectin.

Discussion

In this study, the AL-IVM combination was safe and significantly reduced the survival of 2 major malaria vectors in sub-Saharan Africa, *An. gambiae* and *An. funestus*. The mosquitocidal effect of IVM was apparent 3–7 days after a single dose depending on volunteer sex, with a more pronounced effect when mosquitoes fed on blood from female participants.

The continued move toward malaria elimination has reinvigorated the search for strategies to prevent the spread of malaria, bolstered by a sense of urgency from the threat of artemisinin resistance [4]. Our findings confirm that IVM reduces the life span of different malaria vectors [19], including 2 dominant and important vectors in sub-Saharan Africa, *An. gambiae* and *An. funestus*. Mosquito mortality was 4- to 7-fold increased in mosquitoes that took a blood meal 1 day after IVM. Mosquito mortality was associated with IVM plasma concentrations that decreased markedly during the week after IVM treatment [20]. The waning of the mosquitocidal effect of a single or repeated dose of IVM was slower for female participants, in line with a higher IVM bioavailability in females [21]. The accumulation of IVM in fat tissue [18] and the strong association between BMI and day 7 IVM plasma concentrations suggests that body fat may act as a slow-release reservoir that results in a longer effective half-life of IVM in female participants.

Our findings indicate that higher, repeated doses or sustained presence of drug may be needed for maximal effect. Although IVM is currently recommended as single dose of 200 µg/kg with an excellent safety profile [22], there have been reports where IVM was used repeatedly at higher concentrations [23,24]. We confirmed the tolerability of repeated IVM dosing in a small group of malaria-infected individuals and found no evidence that co-administration of IVM affects the bioavailability of lumefantrine. The primary safety concern for IVM is encephalopathy in individuals heavily infected with microfilariae of *L. Loa* [25] and precludes the use of IVM without prior screening for *L. loa* in endemic areas of Central and West Africa.

Even in individuals with the highest IVM plasma concentrations, the mosquitocidal effect of IVM was not complete and a proportion of mosquitoes survived until day 10. *Plasmodium falciparum* parasites were detected in a small proportion of these surviving mosquitoes. Although our findings do not rule out a sporontocidal effect of IVM, which would require a larger study that is powered for infectivity outcomes, it indicates that malaria transmission potential is not completely abrogated by the AL-IVM combination. If IVM has no impact on gametocytes or their infectivity, its transmission-blocking effect is restricted to its capacity to reduce malaria survivorship in the days immediately following treatment. We estimated that single and repeated doses of IVM may lead to 27% and 35% reductions in post-treatment malaria transmission from symptomatic malaria patients in the first week after treatment with an effective antimalarial. This effect reflects the contribution of an individual patient to malaria transmission and does not reflect population-level impacts that need to take into account effects of IVM on total vector density [8,9,26], reduced mosquito re-feeding rates, and recovery following a blood meal containing sub-lethal doses of IVM [19] and may therefore be larger than reported here. Future studies should further quantify the importance of IVM accumulation in fat tissue for (the duration of) IVM efficacy and be adequately powered to study subtle effects of IVM on sporogonic development. Most important, community trials with repeated doses of IVM are needed to confirm that IVM forms a useful adjunct to reduce and interrupt transmission [27].

In conclusion, our study indicates an incomplete but pronounced effect of IVM on the survival of malaria vectors after IVM ingestion. This effect can be extended by repeated dosing and is associated with the BMI of treated individuals. We observed no evidence for a sporontocidal effect of IVM at mosquito sublethal concentrations in *P. falciparum*-infected individuals. The transmission-blocking properties of IVM may therefore be restricted to its mosquitocidal effects.

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Supporting information 1

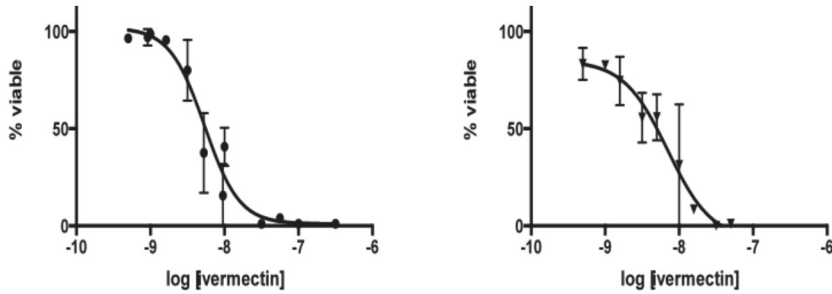
The *Plasmodium falciparum* sporonticidal effect of sublethal concentrations of ivermectin in *Anopheles stephensi* and *An. gambiae* mosquitoes

The clinical trial with artemether-lumefantrine alone or in combination with a single or repeated dose of ivermectin (IVM) observed *P. falciparum* positive mosquitoes that survived until day 10 after feeding on a gametocytaemic blood meal with sublethal concentrations of IVM. Below, we provide supporting information from in vitro experiments where we i) determined the concentration of IVM at which approximately 25% or 10% of *An. stephensi* mosquitoes died within 3 days after feeding (the LC_{25} and LC_{10}); ii) determined oocyst development in *An. gambiae* and *An. stephensi* mosquitoes that were offered cultured gametocytes in combination with ivermectin at a concentration representing the estimated LC_{25} and LC_{10} values.

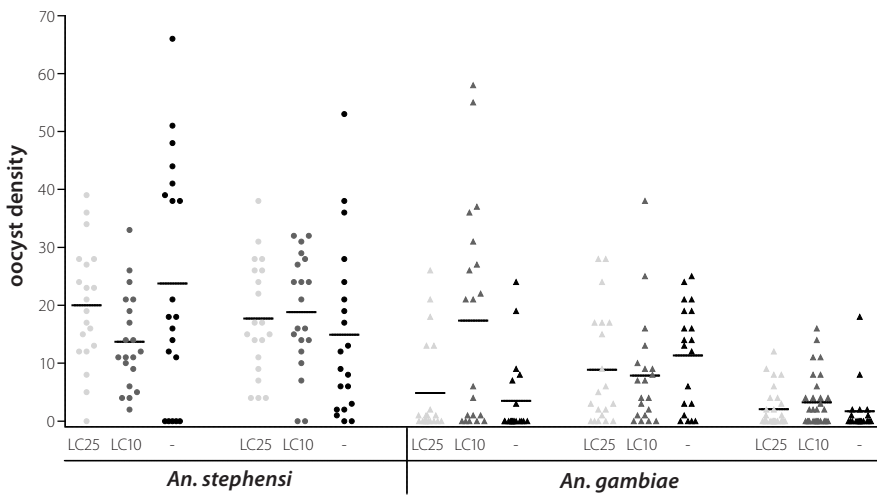
Methods and Results

Anopheles stephensi (Sind-Kasur Nijmegen strain) and *An. gambiae* (Ngousso strain) were reared at 30°C and 70–80% humidity, while exposed to a 12/12 hour day/night cycle as described elsewhere [1]. In initial experiments, mosquitoes were offered IVM in the absence of gametocytes, as described by Kobylinski et al. [2] Powdered IVM [Sigma-Aldrich] was diluted in dimethyl sulfoxide (DMSO) to obtain a 10mM stock (250 mg in 28.57 mL DMSO). This stock concentration was serially diluted in DMSO and subsequently in human serum to reach a final DMSO concentration of 0.1%. The serum containing the diluted compound was mixed with human red blood cells to reach a haematocrit of 50% and fed to *Anopheles* mosquitoes using a glass membrane feeder. After three days, the numbers of dead and live mosquitoes were counted. LC_{25} and LC_{10} values were determined in triplicate experiments (SI Figure 1).

For *An. stephensi* the LC_{25} and LC_{10} were estimated at 3.65×10^{-9} M and 2.2×10^{-9} M by logistic regression. These concentrations were subsequently added to gametocyte culture. For this, mature *P. falciparum* (NF54) gametocytes (14 day culture, 0.3–0.5% gametocytes, 2% haematocrit) were obtained from an automated tipper system and prepared as previously described [3] and offered to mosquitoes using a glass membrane midi-feeder system containing 1.25 mL of the *P. falciparum* culture mix with IVM. Unfed and partially fed mosquitoes were removed after feeding and fully blood-fed females were maintained at 26°C and 70–80% humidity until day 7 post-feeding. Routine staining of midguts was done in 1% mercurochrome after which oocyst densities were quantified. In triplicate replicates in *An. gambiae* and duplicate replicates in *An. stephensi*, there was no difference in oocyst density between surviving mosquitoes in control feeds and feeds with IVM at LC_{25} or LC_{10} (SI Figure 2).



SI Figure 1 The kill curve of ivermectin in *Anopheles stephensi* (left) and *An. gambiae* mosquitoes (right)



SI Figure 2 Oocyst densities in *Anopheles stephensi* (left) and *An. gambiae* (right) mosquitoes using in vitro experiments with NF54 *Plasmodium falciparum* gametocytes and ivermectin

IVM was added to feeders at LC₂₅ and LC₁₀ concentrations; – indicates control feeds with gametocytes but no IVM.

These results indicate that sublethal concentrations of IVM may not affect the development of *P. falciparum* oocysts in surviving mosquitoes.

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Supporting information 2

The estimated net impact of treatment with ivermectin on *Plasmodium falciparum* transmission potential to *Anopheles gambiae* mosquitoes for malaria patients during the first week after treatment with artemether-lumefantrine

In the AL-IVM1 and AL-IVM2 arms, IVM plasma concentrations for 12-hour time windows were estimated based on the dynamics of IVM concentration in the human blood using the following equations,

$$\begin{aligned}\frac{dG}{dt} &= -aG \\ \frac{dB}{dT} &= aG - cB\end{aligned}$$

where G is the concentration of IVM in the gut, B is the concentration of IVM in the blood, a is the rate at which IVM passes from the gut to the blood and c is the rate at which it is cleared from the blood. This model has previously been used to estimate the pharmacokinetic properties of a drug for a range of treatments [1]. We have initial conditions (where is the IVM dose) and .

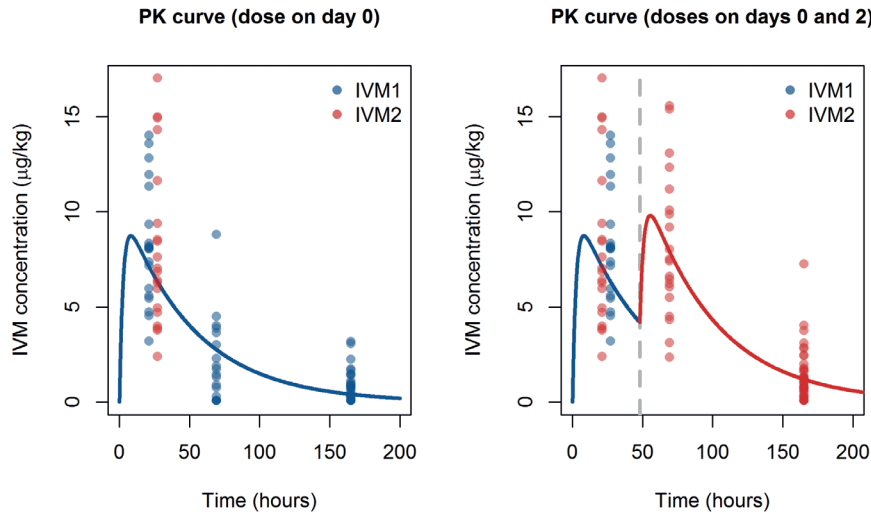
For the first round of treatment this model has the following analytic solutions,

$$B(t) = a G_0 \left(\frac{e^{-ct} - e^{-at}}{a - c} \right). \quad [eq. 1]$$

Parameters a , c , and are estimated by fitting Equation 1 to all the data from IVM1 and to the observations from the first day of IVM2 (Figure S3A). The pharmacodynamics for hours 48–168 in the AL-IVM2 arm are estimated by fitting the analytical solution,

$$B(t) = B_0 e^{-ct} + a G_0 \left(\frac{e^{-ct} - e^{-at}}{a - c} \right) \quad [eq. 2]$$

to the day 3 and day 7 data presented in SI Figure 3B where is the concentration of IVM in the blood as predicted by the first model. Models were fit using maximum likelihood methods.



SI Figure 3 The temporal dynamics of ivermectin concentration in the human blood for a single **(A)** and double **(B)** dose regimen

Points depict observational data whilst lines show the best fit model outlined in Equation 1 and 2 (with parameter values $a = 0.01976$ $c = 0.38535$ and $G0=200 \mu\text{g/kg}$ for A and the initial 48 hours of B, and $a=0.01970$, $b=0.34719$, $G0=200 \mu\text{g/kg}$ and $B0=4.187 \mu\text{g/kg}$ for the latter part of B). Vertical dashed line in (B) signifies the timing of the second treatment. Care should be taken interpreting the early pharmacodynamics of the drug prior to the first data point as this is driven by the structure of the model and has not been confirmed with observational results.

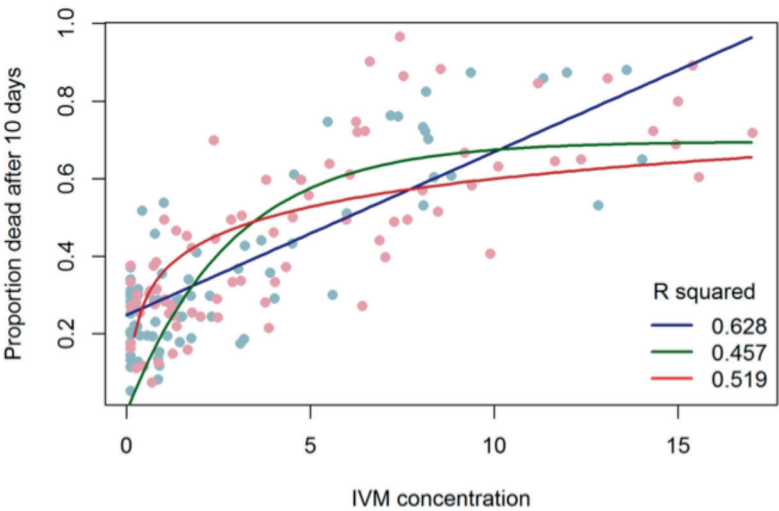
From these fits, the average IVM concentration was determined for 12-hour time periods after single or double-dose IVM (SI Table 1). This was combined with estimates of the relationship between mosquito survival and IVM concentration (as shown by the linear association in SI Figure 4) to generate a prediction of mosquito survival for each time-period following treatment.

SI Table 1 Modelled mean ivermectin plasma concentrations and predicted mosquito survival in the 10 days following a blood meal

It is assumed that IVM is given first thing in the morning and that mosquitoes start feeding 12 hours later (at a constant rate for the next 12-hour period).

Hours		IVM1		IVM2	
		Concentration	Mosquito survival	Concentration	Mosquito survival
0–12	Start treatment	7.24		7.24	
12–24	Average 1 st night	7.51	43.5%	7.51	43.5%
24–36		5.95		5.95	
36–48	Average 2 nd night	4.69	55.4%	4.69	55.4%
48–60		3.70		7.82	
60–72	Average 3 rd night	2.92	62.9%	8.38	39.9%
72–84		2.30		6.65	
84–96	Average 4 th night	1.82	67.5%	5.25	53.0%
96–108		1.43		4.14	
108–120	Average 5 th night	1.13	70.4%	3.27	61.4%
120–132		0.89		2.58	
132–144	Average 6 th night	0.70	72.2%	2.04	66.6%
144–156		0.56		1.61	
156–168	Average 7 th night	0.44	73.3%	1.27	69.8%

IVM, ivermectin.



SI Figure 4 The association between cumulative mosquito mortality and ivermectin plasma concentration

The association between IVM concentration and cumulative mosquito mortality during the 10 days following a blood meal was best described by the equation: mosquito mortality = $0.0421 \text{ (IVM concentration)} + 0.2486$ (blue line, R^2 0.628). Non-linear functions did not improve the fit of IVM concentration and mosquito mortality (green and red line).

These data were combined to estimate the transmission-reducing effect at the level of individual patients (SI Table 2, see explanation in the main text). For this we used gametocyte prevalence and density data from a clinical trial in symptomatic malaria patients [2]. The likelihood that mosquitoes become infected after feeding on a blood-meal with the given gametocyte density was based on a meta-analysis of mosquito feeding data. The analysis was repeated for 2 other datasets [3,4] and did not lead to different conclusions on the comparative effect of IVM1 and IVM2 in comparison with AL monotherapy (data not shown).

SI Table 2 The estimated impact of treatment with ivermectin on *Plasmodium falciparum* transmission from individual malaria patients in the first week after treatment with artemether-lumefantrine

Day of mosquito biting post initial treatment	Gametocyte prevalence (density)*	Proportion of mosquitoes becoming infected†	Proportion of <i>An. gambiae</i> surviving the sporogonic cycle‡			Probability that a susceptible mosquito is infected and survives long enough to become infected (% reduction compared to AL) ^α		
			AL	AL-IVM1	AL-IVM2	AL	AL-IVM1	AL-IVM2
1	79.0 (17.9)	0.142	0.788	0.435	0.43.5	8.8	4.9 (44.5)	4.9 (44.5)
2	61.2 (16.8)	0.140	0.788	0.554	0.55.4	6.8	4.8 (30.2)	4.8 (30.2)
3	43.4(15.7)	0.140	0.788	0.629	0.39.9	4.8	3.8 (20.4)	2.4 (49.5)
4	42.5 (11.6)	0.93	0.788	0.675	0.53.0	3.1	2.7 (13.9)	2.1 (32.4)
5	40.5 (10.0)	0.93	0.788	0.704	0.61.4	3.0	2.7 (11.6)	2.3 (22.9)
6	38.5 (8.4)	0.49	0.788	0.722	0.66.6	1.5	1.4 (9.2)	1.3 (16.2)
7	36.5 (6.8)	0.49	0.788	0.733	0.69.8	1.4	1.3 (6.4)	1.3 (10.8)
Day 1–7 ^β						29.4	21.4 (27.2)	19.0 (35.4)

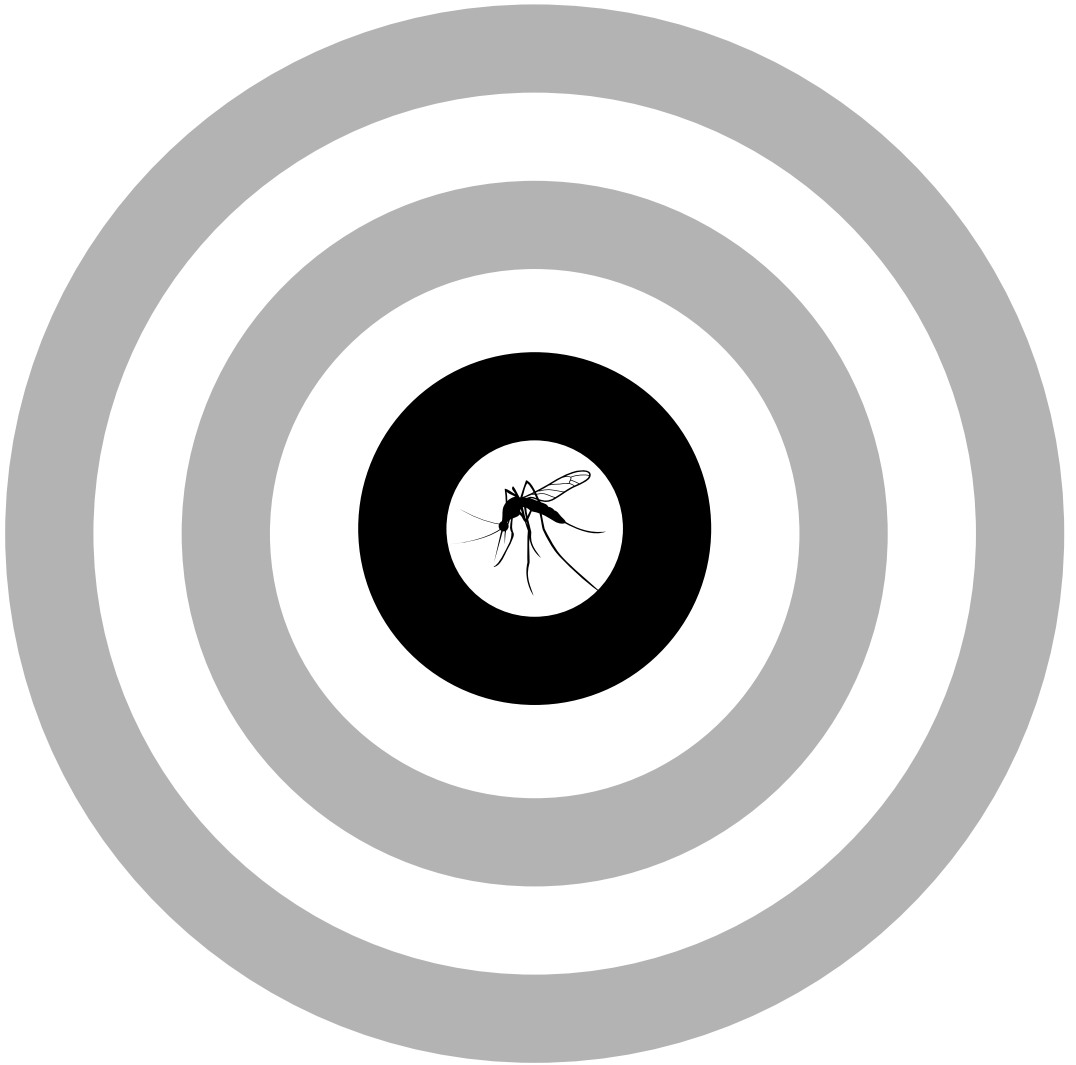
* Gametocyte prevalence and density estimates from Ugandan children aged 1–10 years [2], gametocyte density is shown per μL for gametocyte carriers only; † the probability of mosquito infection at the given gametocyte prevalence and density based on a meta-analysis of mosquito feeding experiments [5] (calculated from $g \times p_{\text{inf}}$, see main manuscript); ‡ For days 1, 3 and 7 mosquito survival rates were directly determined using 4- to 5-day old *An. gambiae* mosquitoes; for days 2, 4, 5 and 6 the IVM concentration was estimated from the best fit of IVM pharmacokinetic data (Figure S3) which in turn was used to estimate mosquito survival (Figure S4). Denoted u_i in the main text. § the relative reduction in transmission from a mosquito biting on a given day following treatment (calculated $100 \times g \times p_{\text{inf}} \times u_i$). Values in brackets are expressed as the total number of mosquitoes completing sporogonic development as a percentage of this summary measure in the AL-only arm). ¶ The sum of the number of mosquitoes completing the sporogonic cycle is a summary measure of transmission potential during 7 days following initiation of treatment; this number can be interpreted as the number of mosquitoes that becomes infected out of 800 mosquitoes that take a blood meal at a rate of 100 mosquitoes per day.

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SECTION C

PROTECTION AGAINST MALARIA BY INOCULATION OF WHOLE SPOROZOITES



CHAPTER 6

PROTECTION AGAINST MALARIA AFTER IMMUNIZATION BY CHLOROQUINE PROPHYLAXIS AND SPOROZOITES IS MEDIATED BY PREERYTHROCYTIC IMMUNITY

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Abstract

Introduction

Volunteers immunized under chloroquine chemoprophylaxis with *Plasmodium falciparum* sporozoites (CPS) develop complete, long-lasting protection against homologous sporozoite challenge. Chloroquine affects neither sporozoites nor liver-stages, but kills only asexual forms in erythrocytes once released from the liver into the circulation. Consequently, CPS immunization exposes the host to antigens from both pre-erythrocytic and blood stages, and induced immunity might target either of these stages. We therefore explored the life cycle stage specificity of CPS-induced protection.

Methods

Twenty-five malaria-naïve volunteers were enrolled in a clinical trial, 15 of whom received CPS immunization. Five immunized subjects and 5 controls received a sporozoite challenge by mosquito bites, whereas 9 immunized and 5 control subjects received an IV challenge with *P. falciparum*-infected erythrocytes. The latter approach completely bypasses pre-erythrocytic stages, enabling a direct comparison of protection against either life cycle stage.

Results

CPS-immunized subjects (13 of 14) developed anticircumsporozoite antibodies, whereas only one volunteer generated minimal titers against typical blood-stage antigens. IgG from CPS-immunized volunteers did not inhibit asexual blood-stage growth in vitro. All CPS-immunized subjects (5 of 5) were protected against sporozoite challenge. In contrast, 9 of 9 CPS-immunized subjects developed parasitaemia after blood-stage challenge, with identical pre-patent periods and blood-stage multiplication rates compared with controls. Intravenously challenged CPS-immunized subjects showed earlier fever and increased plasma concentrations of inflammatory markers D-dimer, IFN- γ , and monokine induced by IFN- γ than IV challenged controls.

Discussion

The complete lack of protection against blood-stage challenge indicates that CPS-induced protection is mediated by immunity against pre-erythrocytic stages. However, evidence is presented for immune recognition of *P. falciparum*-infected erythrocytes, suggesting memory responses unable to generate functional immunity.

Introduction

Malaria remains one of the most common and severe infectious diseases, with an estimated 216 million cases and 655,000 deaths annually [1]. The malaria parasite *Plasmodium falciparum* is responsible for most of these cases, particularly in sub-Saharan Africa. *Plasmodium falciparum* sporozoites are transmitted to humans by the bites of infected *Anopheles* mosquitoes. Sporozoites migrate from the skin to the liver, where they invade hepatocytes, develop, and multiply. Approximately 6 days after invasion, hepatocytes rupture and merozoites are released into the bloodstream, where they multiply in 48-hour cycles of erythrocyte invasion, replication, erythrocyte rupture, and release of infectious merozoites. These asexual blood-stage parasites cause the clinical symptoms of malaria. To fight malaria, an effective vaccine is urgently needed. Development of vaccines generally has been stage-oriented, specifically targeting pre-erythrocytic or asexual blood stages of the parasite [2].

In the controlled human malaria infection model, we previously showed that immunization of healthy malaria-naïve volunteers while they are taking chloroquine prophylaxis with *P. falciparum* sporozoites via infected mosquito bites (ChemoProphylaxis and Sporozoites [CPS] immunization) induces long-lasting sterile protection against a homologous challenge infection [3,4]. The unprecedented efficacy of the CPS immunization model is represented by the low dose sufficient to induce protection, i.e., 3 times 12–15 infected mosquito bites, compared with 1,000 bites required in the irradiated sporozoite approach [5].

Chloroquine kills only developing blood stages of *P. falciparum*, without affecting sporozoites or liver stages [6]. This results in transient low-level blood-stage parasitaemia during CPS immunization [3]. Consequently, the host's immune system will be exposed to a relatively broad repertoire of antigens, including sporozoite, liver-stage, and early blood-stage antigens. Humoral and cellular immune responses are induced against both sporozoites and blood stages [3,7]. In addition, many antigens are shared between these stages [8], leaving open the possibility that the observed protection may be mediated by immune responses against either of these parasite life cycle stages or a combination thereof [9]. The absence of parasitaemia after challenge infection and the predominant induction of pre-erythrocytic antibodies suggest that pre-erythrocytic immunity primarily is responsible for protection, although a possible requirement for immune responses against asexual stages cannot be ruled out [3]. Indeed, previously it was shown that exposure to very low densities of blood stages may induce protection in the controlled human malaria infection model [10]. In this study, protected subjects displayed strong parasite-specific T-cell proliferation and IFN- γ production [10]. Moreover, CPS-immunized volunteers also exhibited strong IFN- γ responses upon in vitro restimulation with infected erythrocytes [7].

To explore the possible role of immunity against the pre-erythrocytic and/or blood stage in protection, CPS-immunized volunteers were subjected to either a *P. falciparum*

sporozoite or an asexual blood-stage challenge. Because the latter approach completely bypasses the liver stages, any protection seen would indicate that blood-stage immunity may contribute to CPS-induced protection.

Methods

Study design

We conducted this single-center, open-label study at the Radboud University Nijmegen Medical Centre (Nijmegen, The Netherlands) from April 2011 until March 2012 following approval by the Central Committee for Research Involving Human Subjects of The Netherlands (CCMO NL34273.091.10). The study team complied with the Declaration of Helsinki and Good Clinical Practice, including monitoring of data. The trial is registered at ClinicalTrials.gov (NCT01236612). Written informed consent of all volunteers was obtained before screening.

Twenty-five healthy subjects (age 18–35 years) without a history of malaria or residence in a malaria-endemic area in the 6 months before study entry were included (Supporting Information [SI] Materials and Methods, Screening of Study Subjects) and randomly assigned to 4 groups (groups 1, 2, 3, and 4; Figure S1). Fifteen subjects received CPS immunization (groups 1 and 2) as described in detail in SI Materials and Methods. Ten controls (groups 3 and 4) received only chloroquine chemoprophylaxis.

Seventeen weeks after discontinuation of chloroquine prophylaxis, corresponding to 21 weeks after the last immunization, all subjects received a challenge infection. Group 1 (n=9; 1 lost to follow-up) and control group 3 (n=5) were challenged by IV administration of 1,962 viable 3D7 *P. falciparum*-infected erythrocytes (blood-stage challenge), which were derived from a stock produced at the Queensland Institute of Medical Research as described previously [11] and used in numerous studies [10–15]. Group 2 (n=5) and group 4 (n=5) were exposed to the bites of 5 3D7 *P. falciparum*-infected *Anopheles stephensi* mosquitoes (sporozoite challenge). Subjects and investigators were aware of the study group, whereas primary outcome assessors were kept blinded to the allocation. All volunteers were treated with a curative regimen of antimalarial drugs at the time of thick smear positivity, or presumptively on day 21 after challenge if thick smears remained negative.

Study outcomes

The primary study outcome was time to parasitaemia after challenge, as assessed by microscopy (SI Materials and Methods). The pre-patent period was defined as the period between challenge and the first positive thick smear. Volunteers were defined as protected from challenge if they remained thick smear negative until day 21. Additionally, parasitaemia was measured retrospectively by quantitative real-time polymerase chain reaction (qPCR) [16]. Blood-stage parasite multiplication rate was calculated as described

previously [17]. Assessment of in vitro growth inhibition and measurements of antibodies, haematological parameters, MIG, and IFN- γ are described in detail in SI Materials and Methods.

Statistical methods

Statistical analysis was performed using GraphPad Prism 5. The difference in AEs among groups was calculated by unpaired Student t test on the accumulative duration of AEs. Differences among groups on the first day of fever ($\geq 37.5^{\circ}\text{C}$), first day of D-dimer increase above 2 times the upper limit of normal ($\geq 1,000$ ng/mL), and first day of detectable IFN- γ and MIG were tested by unpaired Student t test. Differences among groups in pre-patent periods by thick smear and qPCR and blood-stage parasite multiplication rates were tested by the Mann-Whitney test. Differences in antibody levels and in vitro growth inhibition between time-points were tested by paired t test.

Analysis of lymphocyte kinetics after challenge was performed with SPSS version 18 and based on data obtained at days 5, 6, and 7 (pre-treatment). Two regression-type models were fitted to the data. The dependent variable was lymphocyte number, and independent variables were time, treatment, the interaction between time and treatment, and the baseline observation of the dependent variable. The longitudinal character of the data was accommodated using general least-squares estimation; a heterogeneous, unstructured covariance matrix was assumed.

Results

Twenty-five of 42 screened subjects (median age 21 years; range 19–32 years) were included in the study (SI Figure 1). Fifteen volunteers were immunized according to the CPS protocol as described previously [3]. Briefly, while taking chloroquine prophylaxis, volunteers (groups 1 and 2) were exposed to bites of 15 *P. falciparum* infected mosquitoes (8 mosquitoes with the NF54 strain and 7 mosquitoes with the 3D7 clone) at monthly intervals for a period of 3 months. Control volunteers (groups 3 and 4) received chloroquine prophylaxis only. One subject in group 1 withdrew consent after the third immunization for reasons unrelated to the trial.

After the first immunization, 14 of 15 subjects (groups 1 and 2) developed transient low blood-stage parasitaemia, as retrospectively detected by qPCR. The geometric mean of peak parasitaemia was 1,378 parasites/mL (95% confidence interval [CI], 456–4,165 parasite/mL; Figure 1). Thick smears remained negative, except in two subjects (one each in groups 1 and 2) who developed a positive thick smear on day 7. Their peak parasitaemia was 14,454 and 6,761 *P. falciparum*/mL. Both the severity and frequency of adverse events (AEs) were similar to those in the other subjects, and chloroquine plasma concentrations were within the prophylactic range (53 and 56 $\mu\text{g/L}$). These 2 subjects were treated promptly with atovaquone/proguanil and continued study participation according to

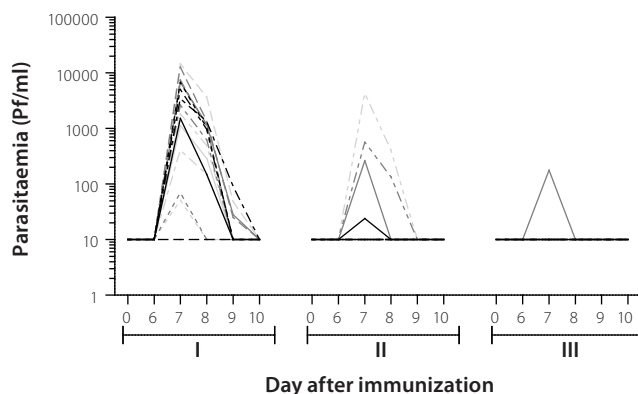


Figure 1 Blood-stage parasitaemia during CPS immunization

Blood-stage parasitaemia was measured from day 6 until day 10 after the first (I), second (II), and third (III) immunization by qPCR. Each line represents an individual subject ($n=15$); values shown as 10 on the logarithmic scale were negative.

protocol. All subjects in groups 1 and 2 reported solicited AEs (mean duration, 1.0 ± 0.11 days) after the first immunization. The most common AEs were headache (13/15 subjects), and fever and nausea (both in 8/15 subjects). Four subjects experienced a grade 3 AE (headache $n=2$, malaise $n=2$; mean duration 1.8 ± 0.6 days), which all occurred between days 7 and 10 after the first immunization and were considered probably related to the immunization.

After the second immunization, 4 subjects developed parasitaemia by qPCR (geometric mean peak parasitaemia, 351 parasites/mL; 95% CI, 43–2,857; Figure 1), whereas thick smears remained negative. Two subjects experienced mild or moderate AEs. After the third immunization, only 1 subject showed blood-stage parasitaemia (178 parasites/mL; Figure 1) and 3 subjects experienced mild AEs. No serious AEs occurred during the trial.

Antibody levels against the circumsporozoite protein (CSP), apical membrane antigen 1 (AMA-1), and glutamate-rich protein (GLURP) were measured before CPS immunization and before challenge. CPS-immunized subjects (13/14) showed induction of anti-CSP antibodies (at least a 2-fold increase in antibody titer), whereas only a single subject (group 1) showed a minimal increase in AMA-1 and GLURP antibody titers (Table 1). Immunoglobulin G (IgG) was isolated from plasma of all immunized subjects at baseline and before challenge infection. In vitro blood-stage growth inhibition assay (GIA) did not show an inhibitory effect of purified IgG on blood-stage parasite growth in any of the subjects (Table 1).

Table 1 Antibody titers and in vitro growth inhibition

Test	I-7	C-1	$\Delta [(C-1) - (I-7)]$	P-value
Antibody titer, AU				
CSP	0.91 (0; 2.15)	24.3 (15.2; 48.5)	24.3 (10.8; 47.3)	0.006
AMA-1	0.12 (0.08; 0.17)	0.11 (0.09; 0.17)	0.00 (-0.03; 0.01)	0.35
GLURP	1.23 (1.01; 2.39)	1.06 (0.90; 3.39)	-0.13 (-0.35; 0.14)	0.46
Growth inhibition, (%)	-3.3 (-7.1; -1.1)	-8.6 (-10.0; -5.46)	-4.4 (-6.0; 1.8)	0.03

Antibody titers against CSP, AMA-1 and GLURP, and in vitro growth inhibitory activity of isolated IgG in all CPS-immunized subjects, before immunization (I-7) and on the day before challenge (C-1). Data are expressed as median (25;75 percentile). Differences between time-points were tested using a paired *t* test. AU, arbitrary units.

The minimum therapeutic plasma chloroquine concentration is 30 µg/L [18], and its reported half-life varies from 5 to 58 days [18,19]. To ensure sufficient clearance of chloroquine in view of the very low blood-stage challenge dose, the challenge infection was conducted 17 weeks after the last chloroquine dose, corresponding to 21 weeks after the last immunization. Group 1 (n=9) and group 3 (n=5) received a blood-stage challenge by IV administration of 3D7 asexual parasites. Group 2 (n=5) and group 4 (n=5) were subjected to a sporozoite challenge using 5 mosquitoes infected with 3D7 sporozoites [20]. There was no difference in parasitaemia between CPS-immunized group 1 and control group 3; both groups became thick smear positive, with a median pre-patent period of 8.0 days (range 7.0–8.3 days and 8.0–8.3 days, respectively; *P*=0.83). Likewise, the pre-patent period by qPCR was similar in both groups (median 5.0 days; range 3.0–5.3 days and 2.0–6.3 days, respectively; *P*=0.41; Table 2). Furthermore, there was no statistically significant difference in multiplication rates of blood-stage parasites between the CPS-immunized subjects and naive controls (median 8 [range 6–18] and 14 [range 7–24], respectively; *P*=0.19; Table 2 and Figure 2).

In group 2, challenged with sporozoites, 4 of 5 CPS-immunized subjects remained negative throughout the follow-up period by both thick smear and qPCR. One CPS-immunized subject, however, showed a positive qPCR at day 21 post-challenge (457 parasites/mL, determined retrospectively). Controls in group 4 all became thick smear positive, with a median pre-patent period of 12.3 days (range 9.3–12.3; multiplication rate 10, range 5–23). Parasitaemia in group 4 (sporozoite-challenged controls) developed approximately 4 days later compared with group 3 (blood-stage-challenged controls; day 5 vs. 9 by qPCR, *P*=0.01; and day 8 vs. 12.3 by thick smear, *P*=0.01; Table 2). The range in pre-patent periods was significantly smaller in group 3 (8.0–8.3 days) than in group 4 (9.3–12.3 days). The parasite multiplication rate in the blood of control subjects was similar

Table 2 Protection against blood-stage versus sporozoite challenge by CPS immunization

	Challenge	Protected/total no. of volunteers	Protection, (%)	Pre-patent period (days) median (range)		Blood-stage parasite multiplication rate, median (range)
				Thick smear	qPCR	
Immunized	Sporozoite	5/5	100	N/A	N/A*	N/A
	Blood-stage	0/9	0	8.0 (7.0–8.3)	5.0 (3.0–5.3)	8 (6–18)
Control	Sporozoite	0/5	0	12.3 (9.3–12.3)	9.0 (7.0–10.0)	10 (5–23)
	Blood-stage	0/5	0	8.0 (8.0–8.3)	5.0 (2.0–6.3)	14 (7–24)

*One subject became qPCR positive on day 21 after challenge. N/A, not applicable.

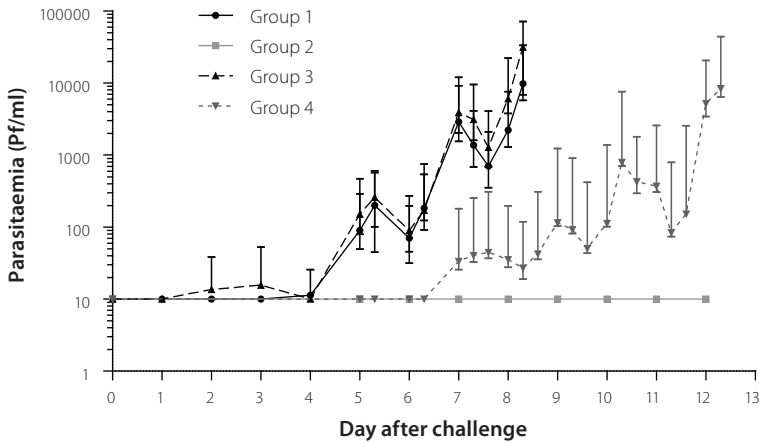


Figure 2 Parasitaemia after challenge as assessed by qPCR

Geometric mean parasite density \pm 95% CI from day of inoculation until the day of treatment after blood-stage challenge [black line, group 1, CPS-immunized (n=9); dashed dark gray line, group 3, controls (n=5)] or sporozoite challenge [light gray line, group 2, CPS-immunized (n=5); dashed light gray line, group 4, controls (n=5)]. Values shown as 10 on the logarithmic scale were negative.

in those receiving either a blood-stage or sporozoite challenge (14 [7–24] vs. 10 [5–23], respectively; $P=0.57$).

All 19 unprotected volunteers reported solicited AEs considered possibly or probably related to the challenge (mean number of AEs per subject, 6.4 ; mean duration, 1.4 ± 0.1 days), including headache, fever, and nausea as the most common symptoms (SI Table 1). The peak of AEs occurred later in subjects who received a sporozoite challenge, concordant with the later onset of parasitaemia, but there was no difference in accumulative duration of AEs compared with blood-stage-challenged controls (Figure 3a1 and a2; $P=0.24$). In contrast, protected subjects showed significantly fewer AEs: 3 of 5 experienced mild or moderate AEs (mean number/subject, 1.4 ; mean duration, 0.3 ± 0.1 days; $P=0.002$ compared with unprotected subjects; Figure 3a2).

Lymphocyte counts decreased after challenge in all unprotected subjects (Figure 4A), as did platelet counts (Figure 4B), with the exception of 1 volunteer. Platelet counts declined below the lower limit of normal ($150 \times 10^9/L$) in 9 of 19 unprotected subjects

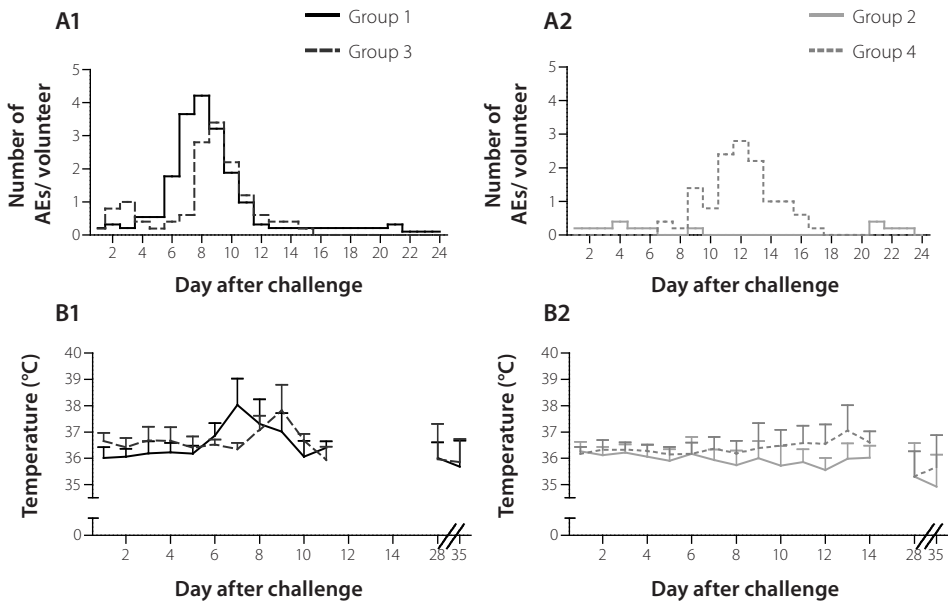


Figure 3 Adverse events and temperature after challenge

Adverse events (AEs) and body temperature were recorded daily after challenge. Blood-stage challenge (**A1**) and sporozoite challenge (**A2**): mean number of possibly or probably related solicited AEs per subject. Blood-stage challenge (**B1**) and sporozoite challenge (**B2**): temperature (mean \pm SEM). Black line, group 1 (CPS-immunized, blood-stage challenge; $n=9$); dashed dark gray line, group 3 (controls, blood-stage challenge; $n=5$); light gray line, group 2 (CPS-immunized, sporozoite challenge; $n=5$); dashed light gray line, group 4 (controls, sporozoite challenge; $n=5$).

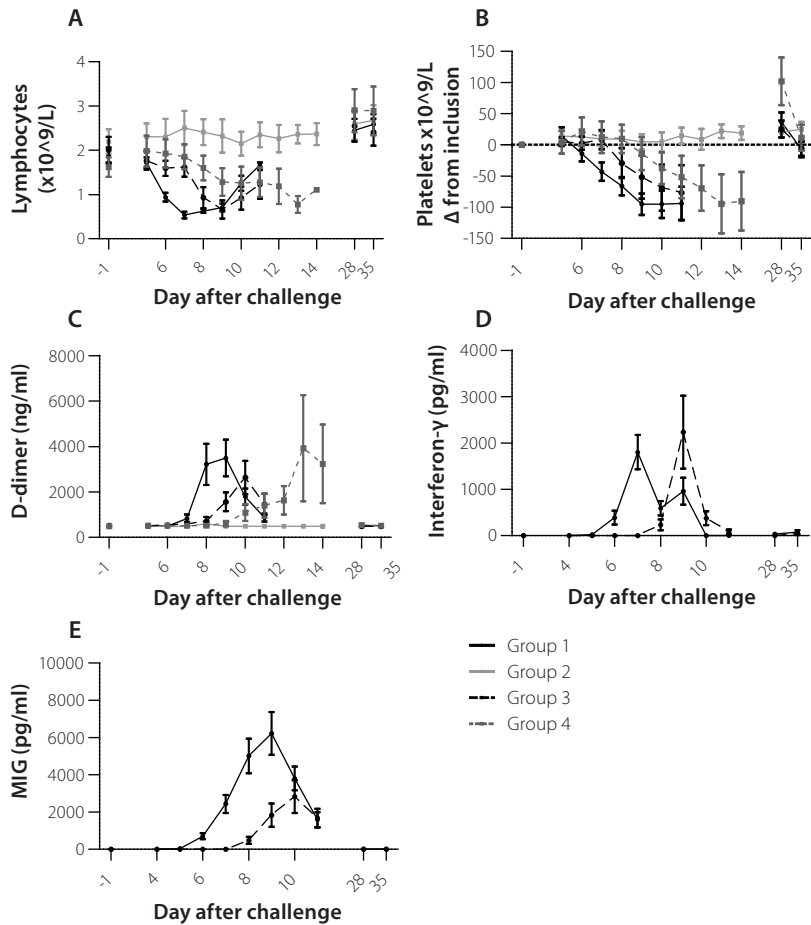


Figure 4 Haematological and inflammatory markers after challenge

(A) Peripheral lymphocyte counts ($\times 10^9/L$). (B) Platelet counts (difference from value at inclusion, $\times 10^9/L$). (C) D-dimer (ng/mL). (D) IFN- γ (pg/mL). (E) MIG (pg/mL). Black line, group 1 (CPS-immunized, blood-stage challenge; $n=9$); dashed dark gray line, group 3 (controls, blood-stage challenge; $n=5$); light gray line, group 2 (CPS-immunized, sporozoite challenge; $n=5$); dashed light gray line: group 4 (controls, sporozoite challenge; $n=5$). Data are shown as mean \pm SEM.

(mean lowest value $132 \pm 10 \times 10^9/L$). D-dimer concentrations were elevated in all thick smear-positive subjects ($n=19$; mean peak concentration, $3,908 \pm 650$ ng/mL; Figure 4C). All abnormal laboratory values normalized without complications; bleeding or thrombotic complications were not detected in any of the subjects.

There was a remarkable difference in occurrence of fever and AEs between blood-stage-challenged groups 1 and 3, although curves of developing parasitaemia were identical (Figures 2 and 3). CPS-immunized subjects in group 1 developed fever at a significantly earlier time-point than controls in group 3 (mean first day of temperature $\geq 37.5^\circ\text{C}$, day 7.25 vs. day 8.5; $P=0.002$; Figure 3B1), concordant with an earlier mean decline in lymphocytes (Figure 4a; $P<0.01$) and increase in D-dimer concentrations (mean first day of D-dimer, $>1,000$ ng/mL, day 8.0 vs. day 9.0; $P=0.05$; Figure 4c).

We next investigated plasma concentrations of IFN- γ , a key mediator of cellular immunity in malaria [21], and monokine induced by IFN- γ (MIG), a downstream mediator in the IFN- γ pathway [22]. Figure 4 D and E shows distinct increases in both IFN- γ and MIG plasma concentrations upon blood-stage challenge at 2–3 days earlier in group 1 compared with group 3 ($P<0.001$).

Discussion

This study shows that sporozoite immunization by *P. falciparum* infected mosquito bites of human subjects while taking chloroquine chemoprophylaxis (CPS immunization) does not protect against an IV administered blood-stage challenge infection. The presence of transient low-level parasitaemia during CPS immunization is sufficient to induce immune recognition of asexual forms, as indicated by an earlier increase of IFN- γ and MIG after blood-stage challenge. However, these responses apparently are insufficient to confer any functional blood-stage immunity. In contrast, complete protection is obtained against a sporozoite challenge by mosquito bites as described before [3,4].

The previously observed absence of detectable parasitaemia in CPS-immunized subjects after mosquito challenge suggested predominance of pre-erythrocytic immunity, but asexual stage immunity might have contributed to protection [3]. In the present study, however, the complete lack of any sign of clinical and/or parasitological protection against even an unphysiologically low blood-stage infection ($<2,000$ ring forms, i.e., 20-fold lower than an estimated average of 40,000 merozoites released from a single infected hepatocyte [23]), suggests the complete absence of any functional blood-stage immunity. This is supported by the lack of antibodies against blood-stage antigens after CPS immunization in all but 1 volunteer, and the absence of in vitro growth-inhibitory activity of IgG isolated from CPS-immunized subjects. The single volunteer who developed detectable, although very low, AMA-1 and GLURP antibody levels was the only subject who experienced qPCR-detectable blood-stage parasitaemia after all three immunizations. One immunized subject developed parasitaemia on day 21 after sporozoite challenge, as retrospectively detected by qPCR. The blood-stage parasite multiplication rate after controlled human malaria infection in malaria-naïve subjects is 10.9 on average, but may be as low as 2 [17]. With a multiplication rate of 2, a load of 457 parasites/mL on day 21 would be the result of an estimated 9,000 merozoites released

from the liver (i.e., one infected hepatocyte). Given the total lack of in vivo protection from blood-stage challenge and in vitro growth-inhibitory activity of IgG in all immunized subjects, the delayed pre-patency in this volunteer most likely was caused by either a profound reduction in liver-stage burden or a prolonged liver stage and therefore delayed release of merozoites into the blood.

Although clinical immunity and control of blood-stage parasitaemia are acquired with repeated parasite exposure in endemic populations, the occurrence of sterile protection mediated by sporozoite/liver stages alone has not been confirmed [24]. Hence, this study is a unique and unambiguous demonstration of induction of sterile pre-erythrocytic immunity generated against non-attenuated wild-type *P. falciparum* sporozoites. Sterile protection induced by immunization with irradiated sporozoites that arrest early after liver cell invasion also most likely is based on pre-erythrocytic immunity [5]. In this situation, asexual forms never occur, and the apparent lack of blood-stage immunity was shown in the 1970s in a single irradiated sporozoite-immunized volunteer challenged with blood-stage parasites [25].

Blood-stage-challenged subjects in our study showed neither a delay in the pre-patent period nor a reduction in asexual multiplication rate compared with naive controls. This is remarkable because several studies have shown that protective immunity to *P. falciparum* blood stages can be induced readily after very few infections [10,26]: (i) Adult patients treated with prolonged *P. falciparum* infections for neurosyphilis in the 1920s and 1930s showed clear evidence of clinical and parasitological immunity during a second asexual blood-stage infection by a decrease in frequency of fever and parasitaemia [26]. (ii) Repeated administration of ± 30 *P. falciparum*-infected erythrocytes followed by early treatment with atovaquone/proguanil induced protection against blood-stage challenge [10] in 3 of 4 subjects, although a potential effect of residual atovaquone blood levels could not be ruled out [27]. A plausible explanation for the absence of blood-stage protection might be the short duration and low grade of parasitaemia as a result of the use of chloroquine and hence insufficient exposure to blood-stage antigens. Induction of protective immunity against blood stages requires several cycles of parasite replication and sufficient duration of parasitaemia [26,28]. Even in the trial by Pombo et al. [10], in which subjects were immunized with unphysiologically low numbers of blood-stage parasites, treatment was initiated only after 8 or 14 days, allowing at least 4 replication cycles and therefore sufficiently long exposure to blood-stage antigens. This stands in contrast to the CPS immunization protocol, in which prophylactic levels of chloroquine constantly are present, preventing a full blood-stage replication cycle of parasites. Thus, although the occurrence of low parasitaemia during CPS immunization might benefit the induction of pre-erythrocytic immunity as a result of the expression of cross-stage antigens, it clearly is insufficient to induce a functional protective immune response against blood stages.

Notwithstanding the absence of protection against blood-stage challenge, we do find evidence for immune recognition of blood stages. Previously, we showed that *P. falciparum*-infected erythrocytes elicit release of IFN- γ , mainly from innate cells including natural killer and $\gamma\delta$ T cells [7,29,30]. This innate response may be enhanced through and supplemented by adaptive memory T cells producing cytokines [31]. In addition, effector memory T cells produce IL-2 and IFN- γ upon in vitro restimulation with *P. falciparum*-infected erythrocytes [7]. In the present study, CPS-immunized subjects, while unprotected against a blood-stage challenge, showed an earlier in vivo peak of plasma IFN- γ in the course of blood-stage infection than naïve controls, despite identical kinetics of developing parasitaemia. Early recognition of blood stages by memory cells in these immunized subjects apparently led to an accelerated and enhanced production of IFN- γ and further downstream mediators, including the chemokine MIG [22]. MIG may have contributed to the observed earlier lymphocyte recruitment out of the peripheral circulation. The clinical and laboratory signs/symptoms in the unprotected CPS-immunized and blood-stage-challenged volunteers (group 1) most likely represent a shift of inflammatory responses common to malaria [32] to earlier time-points compared with the challenged controls. Thus, immune recognition represented by these markers took place at an earlier time-point in CPS-immunized individuals compared with naïve volunteers, suggesting the presence of memory responses to asexual blood stages despite the absence of protection.

Mechanisms and target antigens for protective immunity induced by CPS immunization remain to be unravelled. Although mainly antibodies are important in controlling blood-stage parasitaemia [33], rodent and primate studies indicate that CD8+ T-cell responses against parasite liver stages are critically involved in pre-erythrocytic immunity [34–36]. Therefore, detailed analysis of T-cell responses will be the subject of future studies.

Furthermore, this efficient immunization model will enable studies of antigen specificity of cellular and humoral immune responses for identification of potential new antigens or combinations thereof for subunit vaccine candidates. Malaria vaccine development to date has been stage oriented, aimed at targeting either the pre-erythrocytic or asexual blood-stage of the parasite. Vaccines against asexual blood-stage antigens likely will not prevent infection, but instead may reduce parasite densities and provide protection against clinical disease. Pre-erythrocytic immunization strategies such as CPS immunization, however, induce sterile protection, thereby preventing blood-stage infection [37].

In conclusion, sporozoite immunization by the CPS protocol may induce sterile protection entirely mediated by immune responses against the pre-erythrocytic stages of *P. falciparum*. These findings support a continued focus on vaccine development toward pre-erythrocytic stages, particularly whole-sporozoite approaches.

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Supporting information - Materials and methods

Screening of study subjects

Forty-two subjects without a history of malaria or residence in a malaria-endemic area in the 6 months before study entry were screened for eligibility based on medical and family history, physical examination, and general haematological and biochemical tests. Serologic analyses for HIV, hepatitis B, hepatitis C, and *Plasmodium falciparum* were negative in all subjects. Urine toxicology screening was negative, and none of the volunteers was pregnant or lactating. All subjects had an estimated 10-year risk of developing a cardiac event lower than 5%, as estimated by the systematic coronary evaluation system [1].

Preparation of *P. falciparum*-infected mosquitoes for chemoprophylaxis and sporozoites immunization

Anopheles stephensi mosquitoes were reared at our insectary and infected by allowing them to feed on cultured gametocytes of *P. falciparum* parasites according to standard procedures as described previously [2]. We intended to immunize the subjects with 3D7 parasites only, because this *P. falciparum* strain was to be used for the challenge infections. Initial stocks of 3D7 parasites for immunization and challenge were kindly provided by Adrian Hill, Oxford. However, because of difficulties in culturing 3D7 parasites during the immunization period, we immunized each volunteer with seven 3D7-infected plus eight NF54-infected mosquito bites per immunization instead. The 3D7 strain is a clone of NF54, and both are sensitive to chloroquine. The percentage of infected mosquitoes in the batches used for immunization 1, 2, and 3 was 75, 85, and 90 for 3D7 and 95, 90, and 100 for NF54, respectively. Mosquitoes were infected with an average of 5,042 and 109,000 sporozoites for 3D7 and NF54, respectively.

Chemoprophylaxis and sporozoites immunization procedure

Chloroquine was administered to all subjects according to a standard prophylactic regimen for a period of 14 weeks (98 days) as described previously by Roestenberg et al. [3]. While receiving chloroquine, groups 1 and 2 (15 subjects) were immunized 3 times at monthly intervals, starting 8 days after the first chloroquine dose. Immunization was performed by exposure to the bites of exactly 15 *P. falciparum*-infected mosquitoes for 15 minutes, briefly interrupted twice. Following each feeding session, the salivary glands of all blood-engorged mosquitoes were dissected to confirm the presence of sporozoites. All 10 control subjects (groups 3 and 4) received chloroquine prophylaxis only, but no mosquito bites. On days 6–10 after each immunization, we checked all 15 immunized subjects once daily at our outpatient clinical research department. Blood was drawn for thick blood smears, standard haematological markers, and retrospective assessment of blood-stage parasitaemia by qPCR using *P. falciparum* standard curves prepared by DNA extraction from titrated samples of ring-infected cells [4]. All signs and symptoms were

recorded by the attending physician as mild (grade 1, easily tolerated), moderate (grade 2, interferes with normal activity), or severe (grade 3, prevents normal activity), or in the case of fever, grade 1 (>37.5 – 38.0°C), grade 2 (>38.0 – 39.0°C), or grade 3 ($>39.0^{\circ}\text{C}$). For safety reasons related to a previously reported cardiac event [5], we measured cardiovascular markers throughout the trial (highly sensitive troponin, platelets, D-dimer, and lactate dehydrogenase). Whenever abnormal, blood samples were checked for the presence of fragmentocytes and von Willebrand-cleaving protease activity, according to previous protocols [6].

Challenge infections

Seventeen weeks after discontinuation of chloroquine prophylaxis, corresponding to 21 weeks after the last immunization, all subjects received a challenge infection. Group 1 ($n=9$; 1 lost to follow-up) and control group 3 ($n=5$) subjects were challenged by IV administration of 3D7 *P. falciparum*-infected erythrocytes (blood-stage challenge), whereas group 2 ($n=5$) and group 4 ($n=5$) were exposed to the bites of 5 3D7 *P. falciparum*-infected *An. stephensi* mosquitoes (sporozoite challenge). Subjects were checked daily on an outpatient basis for symptoms and signs of malaria, thick blood smears, hematologic tests, and cardiovascular markers. Subjects who received a blood-stage challenge were checked from the first day after challenge onward, sporozoite-challenged subjects from day 5 onward. All signs and symptoms (solicited and unsolicited) were recorded as described for the chemoprophylaxis and sporozoites (CPS) immunization. At the time of thick smear positivity, each subject was treated with a curative regimen of 1,000 mg atovaquone and 400 mg proguanil once daily for 3 days according to Dutch national guidelines. Subjects who remained thick smear negative until day 21 after challenge were treated presumptively with the same curative regimen. Complete cure was confirmed by 2 consecutive parasite-negative blood smears after treatment.

Blood-stage inoculum

Inocula for blood-stage challenge were derived from a stock of 3D7 *P. falciparum*-infected erythrocytes (blood group O, rhesus negative) produced at the Queensland Institute of Medical Research, as described previously [7]. The donor was seronegative or PCR negative, or both, for a panel of parenterally transmissible viruses at the time of donation and 1 year later, but was IgG positive for EBV and CMV [7]. The blood-stage inoculum was cryopreserved in Glycerolyte 57 and stored in liquid nitrogen under temperature-monitored conditions. To prepare the inocula for IV administration, an aliquot of the seed stock was thawed and washed under sterile conditions as described previously [8] using solutions licensed for clinical use and sterile, single-use consumables. Bacterial culture plates of the hood and both aerobic and anaerobic blood culture of the inoculum did not show any bacterial growth. The inoculum was prepared by diluting to the appropriate dose and dispensed aseptically into 5-mL syringes for administration. Based on microscopic

estimates of the donor's parasite density before freezing of blood samples, each inoculum contained 4,289 infected erythrocytes. The inocula were kept on ice during preparation. Alternating between CPS-immunized and control subjects, all volunteers were inoculated IV between 103 and 111 minutes after thawing of the inocula. The number of viable parasites in the inoculum was verified in retrospect by limiting dilution culture and qPCR detection as previously described [4,8], and the inoculum was calculated to contain 1,962/4,289 (46%) viable/total parasites per subject. This recovery of viable parasites was in the range of what has been reported for the inoculum elsewhere [8-10].

Sporozoite challenge

Anopheles stephensi mosquitoes fed on cultured gametocytes of *P. falciparum* strain 3D7 were 100% infected with an average of 100,000 sporozoites per mosquito. A total of 10 volunteers from group 2 (n=5) and group 4 (n=5) were exposed to bites of 5 infected *An. stephensi* mosquitoes for 10 minutes, as described previously [3]. One feeding session was sufficient for 5 volunteers, whereas a second session was required in the remaining 5 volunteers to obtain an infectious challenge by exactly 5 infected mosquitoes in all 10 subjects.

Study outcomes

The primary study outcome was time to parasitaemia after challenge, as assessed by microscopy. Sampling started on a daily basis for groups 1 and 3 (blood-stage challenge) on days 1–4 and continued for all groups post-challenge twice daily on days 5 and 6, thrice daily on days 7–11, again twice daily on days 12–15, and finally once daily on days 16–21. Thick blood smears were made from 15 µL of EDTA-anticoagulated blood spread over the standardized surface of 1 well of a 3-well glass slide according to harmonized standard protocol for Controlled Human Malaria Infections (Laurens MB, Roestenberg M, and Moorthy VS; manuscript in preparation). After drying, wells were stained with Giemsa for 30 minutes. Slides were read at 1,000x magnification by assessing 200 high-power fields, equal to about 0.5 µL of blood. The smear was deemed positive if 2 unambiguously identifiable parasites were found. Lymphocyte and platelet counts were determined in EDTA-anticoagulated blood with the Sysmex XE-5000. D-dimer concentrations were assessed in citrate plasma by STA-R (Roche).

Immunological measurements

Concentrations of malaria antigen specific antibodies were determined in serially diluted citrate plasma by standardized ELISA in Nunc MaxiSorp plates (Thermo Scientific) coated with 1 µg/mL full-length circumsporozoite protein (CSP), apical membrane antigen 1 (AMA-1; FVO-allele) [11], or glutamate-rich protein (GLURP) [12] diluted in PBS. ELISAs were developed using biotinylated polyclonal goat antihuman IgG (1/2,500), streptavidin-conjugated HRP (1/2,000), and tetramethylbenzidine (all from Mabtech). Spectrophoto-

metrical absorbance was measured at 450 nm. Levels of antibody reactivity were analyzed in relation to a pool of sera from adults living in a highly endemic area in Tanzania [3], which was defined to contain 100 arbitrary units (AU) of IgG directed against an antigen. Antibody responses were considered positive when they had increased at least 2-fold after immunization, compared with baseline. Antibodies for growth inhibition assay (GIA) were isolated from citrate plasma collected the day before the start of chloroquine prophylaxis and the day before challenge infection. IgG was purified using protein G columns (HiTrap™ Protein G HP, GE Healthcare). Eluted samples were exchanged into RPMI-1640 on a Vivaspin 20 30-kDa molecular mass cut-off ultrafiltration unit (Sartorius), concentrated to 20 mg/mL, filter sterilized, and stored at -20°C until used. IgG protein concentrations were measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). The GIA was performed as previously described [11]. Briefly, the effect of IgG on in vitro parasite growth was evaluated at an IgG concentration of 10 mg/mL. Samples were run in triplicate using alanine-synchronized cultures of *P. falciparum* 3D7 schizonts at an initial parasitaemia of 0.7%. After 40–42 hours, parasite growth was assessed by measuring parasite lactate dehydrogenase levels in culture supernatants with the lactate diaphorase 3-acetylpyridine adenine dinucleotide substrate system. EDTA (4 mM) was included in every test plate as a positive control. Induction of growth inhibitory activity by CPS immunization was assessed by subtracting for each volunteer the pre-immunization percentage of growth inhibition from the post-immunization value. Monokine-induced-by-IFN- γ (MIG) and IFN- γ concentrations in frozen and stored EDTA anticoagulated plasma samples were determined retrospectively. MIG was measured using a DuoSet ELISA (R&D Systems) and IFN- γ was measured using a Ready-SET-Go! ELISA kit (eBioscience) according to the manufacturer's recommendations.

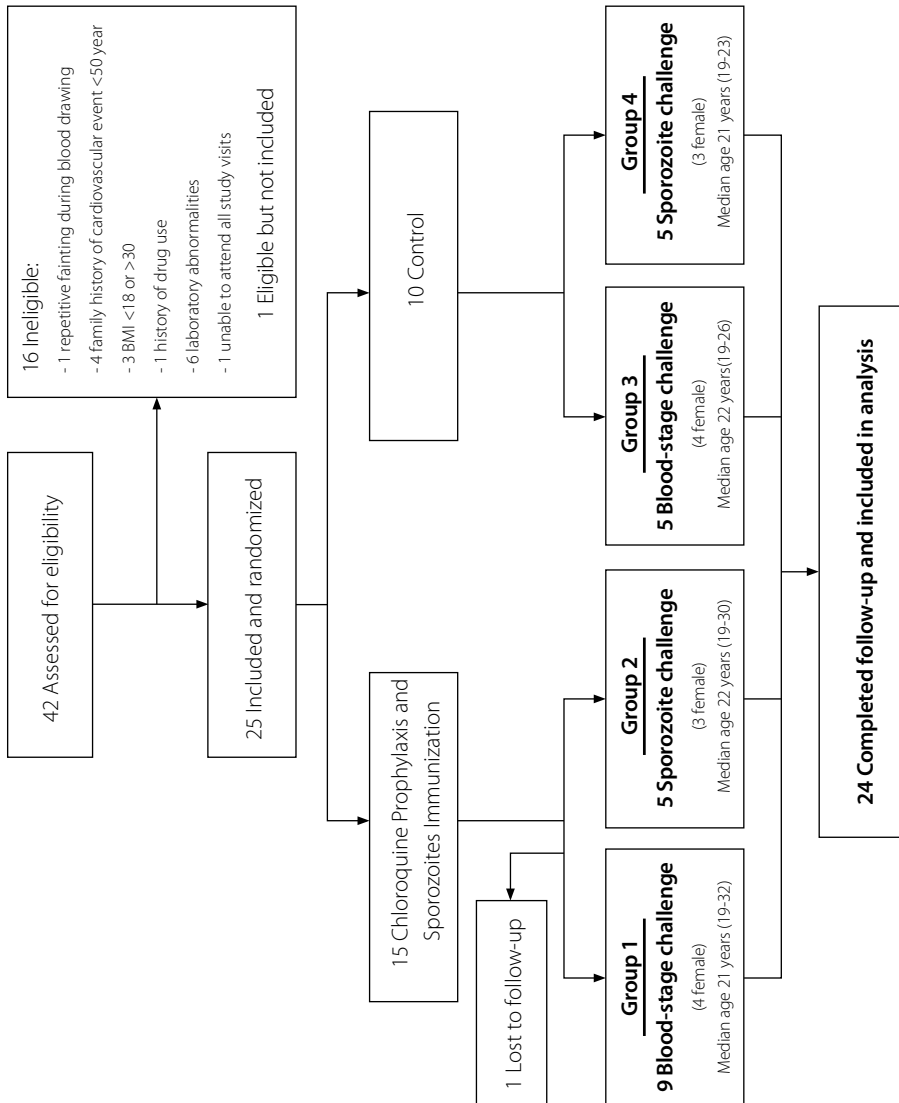


Figure S1 Study flow diagram with relevant baseline data

Fifteen subjects were CPS-immunized by the bites of 3×15 *P. falciparum*-infected mosquitoes while taking chloroquine prophylaxis (groups 1 and 2). Ten control subjects received chloroquine prophylaxis but no infected mosquito bites (groups 3 and 4). One subject withdrew informed consent after the immunization for reasons unrelated to the trial. Seventeen weeks after discontinuation of chloroquine prophylaxis, nine immunized (group 1) and five control volunteers (group 3) received a blood-stage challenge, whereas five immunized (group 2) and five control volunteers (group 4) received a sporozoite challenge by mosquito bites.

SI Table 1 Possibly or probably related solicited adverse events after challenge infection

Adverse event	Blood-stage challenge			Sporozoite challenge		
	CPS-immunized (n=9)		Control (n=5)	CPS-immunized (n=5)		Control (n=5)
	No. of volunteers	Mean duration \pm SD (days)	No. of volunteers	Mean duration \pm SD (days)	No. of volunteers	Mean duration \pm SD (days)
Any						
Abdominal pain	3	1.5 \pm 0.4	2	0.6 \pm 0.3	0	N/A
Arthralgia	1	1.6	0	N/A	0	N/A
Chills	2	0.8 \pm 0.0	0	N/A	1	0.0
Fatigue	3	2.2 \pm 4.3	2	2.8 \pm 0.3	0	N/A
Fever	8	1.4 \pm 0.6	5	1.6 \pm 1.0	1	0.3
Headache	8	1.6 \pm 0.9	5	1.4 \pm 1.2	2	1.4 \pm 1.1
Malaise	3	2.3 \pm 1.4	0	N/A	0	N/A
Myalgia	6	1.6 \pm 0.9	3	2.9 \pm 2.7	0	N/A
Nausea	8	0.9 \pm 0.6	4	1.8 \pm 1.5	2	0.4 \pm 0.6
Vomiting	4	0.9 \pm 0.8	1	1.4	0	N/A
Any	9	1.6 \pm 1.5	5	1.7 \pm 0.8	3	0.8 \pm 1.4
Grade 3						
Abdominal pain	1	1.8	0	N/A	0	N/A
Arthralgia	0	N/A	0	N/A	0	N/A
Chills	0	N/A	0	N/A	0	N/A
Fatigue	0	N/A	1	3.0	0	N/A
Fever	4	1.3 \pm 0.7	0	N/A	0	N/A
Headache	1	1.0	2	1.2 \pm 1.0	0	N/A
Malaise	3	2.3 \pm 1.7	0	N/A	1	2.0
Myalgia	0	N/A	0	N/A	0	N/A
Nausea	6	0.9 \pm 0.5	1	0.8	0	N/A
Vomiting	4	0.9 \pm 0.8	1	1.4	0	N/A
Any	8	1.2 \pm 0.9	3	1.5 \pm 1.0	0	N/A
					3	1.3 \pm 1.3

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CHAPTER 7

SAFETY, IMMUNOGENICITY AND PROTECTIVE EFFICACY AFTER INTRADERMAL IMMUNIZATION WITH ASEPTIC, PURIFIED, CRYOPRESERVED *PLASMODIUM FALCIPARUM* SPOROZOITES IN VOLUNTEERS UNDER CHLOROQUINE PROPHYLAXIS: A RANDOMIZED CONTROLLED TRIAL

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in preparation

Abstract

Introduction

Our previous studies in the Controlled Human Malaria Infection (CHMI) model show that volunteers develop complete, long-lasting protection against a standard homologous *Plasmodium falciparum* (Pf) infection when immunized under chloroquine chemoprophylaxis by bites of sporozoite (SPZ)-infected mosquitoes. Here, we studied the safety, tolerability and induction of protective efficacy against homologous Pf CHMI after intradermal (ID) immunization with aseptic, purified, cryopreserved, infectious PfSPZ (NF54 strain) in volunteers taking weekly chloroquine chemoprophylaxis (PfSPZ-CVac).

Methods

Thirty healthy malaria-naïve volunteers were enrolled in a double-blind, placebo controlled trial. All volunteers received chloroquine chemoprophylaxis during the immunization period. Vaccine group 1 (n=10) received 3 immunizations at 4 week intervals with 75,000 PfSPZ (total 225,000) administered by 6 ID 10 µL injections at each session while control group 2 (n=5) received normal saline administered in the same fashion. Protective efficacy was tested by CHMI at 60 days after the last immunization. Vaccine group 3 (n=8) received 4 rounds of ID immunizations with 75,000 PfSPZ (total 300,000) with group 4 (n=5) as normal saline control; 4 subjects of both group 3 and 4 were subjected to CHMI at 137 days after the last immunization.

Results

Similar percentages of PfSPZ-CVac recipients (11/20, 55%) and vaccine controls (6/10, 60%) reported mild to moderate probably/possibly solicited adverse events (AEs) and no serious AEs during immunization. A total of 23 volunteers were subjected to CHMI; 2/10 subjects in group 1 remained aparasitaemic by microscopy but not by qPCR during the follow-up period of 21 days, and a parasite killing effect of residual chloroquine levels cannot be excluded. All other volunteers that received CHMI (n=21) developed parasitaemia with no significant difference in pre-patent period between both vaccine groups 1 (n=8) and 3 (n=4) as well as control groups 2 (n=5) and 4 (n=4). One subject in vaccine group 1 developed a cardiac serious adverse event (SAE) after CHMI as reported elsewhere. Only weak humoral responses assessed by PfSPZ immunofluorescence assay and specific ELISAs for PfCSP, PfLSA1 and PfMSP1 were induced in vaccine group 1 and 3, while there were no *in vitro* cellular re-call responses against NF54 parasites as determined by flow cytometry.

Discussion

ID immunization with up to a total of 300,000 PfSPZ-CVac was safe and well tolerated, but induced only minimal immune responses and subsequent protection if any against a homologous CHMI. Dose and/or route of PfSPZ-CVac administration were insufficient and unable to induce the immune responses required for protection.

Introduction

Malaria accounts for 207 million clinical cases a year worldwide and an estimated 627,000 deaths, with children under 5 years of age in sub-Saharan Africa most severely affected [1]. Significant advances have been made in malaria control with the number of confirmed cases steadily reducing and mortality rates dropping by 49% in the World Health Organization (WHO) African Region between 2000 and 2012 [1]. To ensure these positive trends and maintain gains achieved over the past decade, current control and preventive measures such as artemisinin-based combination therapies (ACTs), rapid diagnostic tests (RDTs), long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) should be supported by a highly-effective malaria vaccine. Emergence of artemisinin-resistant malaria in Southeast Asia [2,3] and widespread insecticide resistance in malaria transmitting anopheline mosquitoes [4] further increase this need. Combining various control and preventive measures including large-scale vaccination will ultimately offer the best prospect for success.

Progress in the clinical development of efficient immunization strategies as a forerunner of an effective malaria vaccine has been facilitated by controlled human malaria infections (CHMIs). CHMIs involve small groups of malaria-naïve volunteers exposed to the bites of *Plasmodium falciparum* (Pf) sporozoite (SPZ)-infected laboratory-reared *Anopheles* mosquitoes. We have previously shown that healthy malaria-naïve volunteers can be fully protected against a mosquito CHMI with a homologous Pf strain for more than two years after three immunizations under chloroquine prophylaxis by bites from 12-15 PfSPZ-infected mosquitoes at monthly intervals (ChemoProphylaxis and Sporozoites, CPS) [5,6]. Protection is mediated by immunity against pre-erythrocytic stages [7].

While a strong proof of concept, this protocol is unsuitable for direct practical application as long as PfSPZ are inoculated by mosquito bites. Sanaria Inc. (Rockville, USA) has developed a process for manufacturing infectious, aseptic, purified, vialled, cryopreserved PfSPZ (PfSPZ Challenge) in compliance with all regulatory standards [8-11]. To date, single doses of cryopreserved PfSPZ have been administered at different doses up to 125,000 PfSPZ in 184 human subjects by the intradermal (ID) (n=84), intramuscular (IM) (n=70), or direct venous inoculation (IV) (n=30) routes using a needle and syringe in order to assess safety, tolerability and infectivity [8,9,11].

Here, we report the first phase 1 trial of CPS immunization with cryopreserved PfSPZ, the PfSPZ-CVac (PfSPZ-ChemoProphylaxis Vaccine) approach to assess, safety, immunogenicity and protection against CHMI with Pf-infected mosquitoes.

Methods

Study population

We recruited healthy male and female subjects aged 18 to 35 years, adhering to inclusion and exclusion criteria as described previously [7]. All subjects had an estimated 10-year risk of developing a cardiac event smaller than 5% as estimated by the systematic coronary evaluation system [12]. Baseline ophthalmologic examination revealed no abnormalities on fundoscopy that might preclude treatment with chloroquine.

Subjects gave written informed consent before inclusion. The trial was conducted in accordance with Good Clinical Practice and approved by the Central Committee for Research Involving Human Subjects of The Netherlands (CCMO NL39541.091.12). An Investigational New Drug application was filed with the U.S. Food and Drug Administration. Clinicaltrials.gov identifier: NCT01728701.

Trial design

This prospective, single centre, double-blind, randomized, placebo-controlled clinical trial was performed at the Radboud university medical center (Radboudumc), Nijmegen, The Netherlands, from September 2012 to February 2014. Thirty subjects were randomly assigned to 4 study groups: vaccine groups 1 and 3 (each 10 subjects) and control groups 2 and 4 (each 5 subjects) (Figure 1). All groups received injections with either cryopreserved PfSPZ (strain NF54) or normal saline (NS) under chloroquine cover as described below. Sixty days after the last immunization with cryopreserved PfSPZ, groups 1 and 2 received a standard CHMI by 5 mosquitoes infected with Pf NF54 SPZ [13]. Protection was defined as thick smear negative through day 21 post-CHMI. Subsequent study procedures involving groups 3 and 4 were dependent on the rate of protection: if $\geq 75\%$, groups 3 and 4 would receive CHMI with heterologous Pf NF135.C10-infected mosquitoes [14]; if protection rate proved to be $< 75\%$, a fourth PfSPZ-CVac immunization would follow with subsequent homologous Pf NF54 CHMI.

PfSPZ-CVac immunizations

All subjects received standard chloroquine chemoprophylaxis for a period of 14 weeks (98 days) as described previously [5]. On days 8, 36 and 64 following initiation of chloroquine chemoprophylaxis, vaccine groups received 6 intradermal (ID) 10 μ L injections (in the deltoid region of both arms) of PfSPZ Challenge, containing a total of 75,000 cryopreserved PfSPZ of the Pf NF54 strain. Controls received 6 ID 10 μ L injections of NS. The vialled cryopreserved PfSPZ in liquid nitrogen vapour phase were thawed and diluted in phosphate buffered saline containing 1% human serum albumin and all subjects were injected within 30 minutes of thawing. In addition subjects in groups 3 and 4 received a fourth injection of PfSPZ Challenge or NS 168 days after the third immunization.

On days 5, and 10 to 14 after injections, subjects were checked on an outpatient basis by attending physicians and blood was drawn for thick blood smears, standard haematological and biochemical parameters, markers of myocardial tissue damage, coagulation, inflammation, and hemolysis as described previously [7], and retrospective assessment of blood-stage parasitaemia by quantitative real-time polymerase chain reaction (qPCR). Additional blood samples for qPCR measurements were provided on a voluntary base on days 8 and 9 after the second and third immunizations. All signs and symptoms (solicited and unsolicited) were recorded and graded as follows: mild/grade 1 (awareness of symptoms that are easily tolerated and do not interfere with usual daily activity), moderate/grade 2 (discomfort that interferes with or limits usual daily activity), or severe/grade 3 (disabling, with subsequent inability to perform usual daily activity, resulting in absence or required bed rest). Tympanic temperature was measured and recorded as fever grade 1 (37.6–38.0°C), grade 2 (>38.0–39.0°C), or grade 3 (>39.0°C). Causality of adverse events (AEs) was classified as not-related, possibly, probably or definitely related to the trial.

CHMI by mosquito bite

Anopheles stephensi mosquitoes were reared at the Radboudumc insectary and infected by feeding on cultured gametocytes of Pf N54 parasites, according to standard procedures as described previously [15]. The percentage infected mosquitoes of the batches used for challenge infection was 100, and mosquitoes were infected with an average of 75,800 and 98,000 PfSPZ per mosquito for CHMI #1 (groups 1 and 2) and CHMI #2 (groups 3 and 4), respectively.

Thirty-three days after the last dose of chloroquine, corresponding to 60 days after the last immunization with cryopreserved PfSPZ, vaccine group 1 (n=10) and control group 2 (n=5) underwent CHMI by the bites of 5 *An. stephensi* mosquitoes infected with the Pf NF54 strain for 10 minutes as described previously [13]. The salivary glands of all blood-engorged mosquitoes were dissected to confirm the presence of PfSPZ. When necessary, feeding sessions were repeated with fewer mosquitoes until exactly 5 infected mosquitoes had fed. Starting from day 5 after CHMI, subjects were checked daily on an outpatient basis as described above for PfSPZ-CVac immunizations. Blood sampling for thick smear reading and retrospective assessment of parasitaemia by qPCR was performed once daily on days 5 and 6, twice daily on days 7–15, once daily on days 16–21, and for 2 days after initiation of antimalaria treatment for positive thick smears. Antimalaria treatment, consisting of a curative regimen of Malarone (1000 mg atovaquone and 400 mg proguanil) once daily for 3 days, was initiated either as soon as parasites were detected on a thick blood smear, or 21 days after CHMI by mosquito bite for those who did not become infected. Final follow-up visits were on days 35 and 140 after CHMI by mosquito bite.

On day 14 after CHMI 1 subject in vaccine group 1 was unblinded due to a cardiac serious adverse event (SAE) [16]. All other subjects in groups 1 and 2 were unblinded according to protocol 24 days after CHMI by mosquito bite.

Due to the cardiac SAE, the trial was put on hold for 64 days (13 March 2013–16 May 2013) by the Safety Monitoring Committee and the Central Committee for Research Involving Human Subjects of The Netherlands. New safety measures were adopted for follow-up after mosquito CHMI of groups 3 and 4. The endpoint of thick blood smear positivity for diagnosis of malaria was changed to qPCR positivity. Malarone treatment was to be initiated after i) 2 consecutive positive qPCRs when temperature $<38.0^{\circ}\text{C}$, ii) 1 positive qPCR in the presence of a temperature $\geq 38.0^{\circ}\text{C}$, or iii) a positive thick smear prepared upon indication during the evening visit. Subjects who underwent standard vaccinations within 3 months before start of the trial or were planning to take standard vaccinations during the trial period up to 8 weeks after CHMI were excluded.

Study outcome parameters

The primary study outcome was frequency and magnitude of adverse events. Secondary study outcomes included occurrence of Pf parasitaemia after each immunization and CHMI, as assessed by microscopy of thick blood smears and/or qPCR. Thick blood smears were prepared and read as described previously [7]. qPCR was performed as described previously [17] with some modifications. Briefly, 5 μL Zap-oglobin II Lytic Reagent (Beckman Coulter, ref no: 7501369-HA) was added to each 0.5 mL blood sample, mixed and stored at -80°C . After thawing samples were spiked with Phocine Herpes Virus (PhHV) as extraction control and DNA was extracted by a MagnaPure LC isolation instrument. Isolated DNA was re-suspended in 50 μL H_2O and 5 μL was used as template. For the detection of Pf the TaqMan MGB probe AAC AAT TGG AGG GCA AG-FAM was used. For the quantification of PhHV we used primers and probe as described previously [18]. The sensitivity of qPCR was 35 parasites/mL of whole blood. The prepatent period (by qPCR or thick smear) was defined as the period between mosquito bite CHMI and the first positive qPCR (≥ 500 parasites/mL) or thick smear result (≥ 2 unambiguous parasites). The difference in the pre-patent period assessed by qPCR compared to thick smear was defined as Δ pre-patency.

Immunologic analysis

Plasma and peripheral blood mononuclear cells (PBMCs) were obtained from blood collected into citrated BD Vacutainer CPT Cell Preparation Tubes on the following time-points: 1 day before initiation of chloroquine prophylaxis (I1-1) for all volunteers, the day before the third immunization (I3-1) and CHMI (C-1, 59 days after the third immunization) in groups 1 and 2, and 1 day before and 52 days after the fourth injection of PfSPZ/ NS injections in groups 3 and 4 (I4-1, I4+52).

Analysis of antibody responses by ELISA

Plasma concentrations of malaria antigen-specific antibodies were determined against a pool of 100 sera from adults living in a highly endemic area in Tanzania (HIT serum [5]) by standardized enzyme-linked immunosorbent assay (ELISA). Recombinant proteins of circumsporozoite protein (PfCSP) and liver stage antigen 1 (PfLSA-1) [19] represent sporozoites and liver stages, respectively, while merozoite surface protein 1 recombinant protein (PfMSP-1) [20] was used to assess reactivity against late liver and blood stages [21].

96-well Polystyrene flat-bottom plates (NUNC™ Maxisorp, Thermo Scientific) were coated overnight at 4°C with 2 µg/mL of antigen, washed with PBS and blocked for 1.5 hours at room temperature (RT) with 150 µL of 5% milk in PBS. In all the washing steps that followed, plates were washed with PBS + 0.05% Tween (PBST). Serially diluted plasma samples (starting at 1:50 to 1:800 in 1% milk in PBST [sample buffer]) were incubated for 3 hours at RT in a humidified chamber. As a standard, duplicates of pooled HIT serum were included on every plate in a 7-point dilution series. Reactivity for each antigen in undiluted HIT serum was defined as 100 arbitrary units (AU). Bound immunoglobulin G (IgG) was detected using horseradish peroxidase (HRP) conjugated anti-human IgG (Thermo Scientific) followed by TMB One Component HRP Microwell Substrate (tebu-bio). The reaction was stopped using 0.2M H₂SO₄ and absorbance measured with a spectrophotometer plate reader at 450 nm (Anthos 2020 ELISA plate reader). Optical density (OD) values were converted into AUs by four-parameter logistic curve fit using Auditable Data Analysis and Management System for ELISA (ADAMSEL-v1.1, <http://www.malariaearesearch.eu/content/software>). An increase in AU of more than 1 was considered an actual increase.

Analysis of *Plasmodium falciparum* sporozoite immunofluorescence assay

Two x10³ purified PfSPZ suspended in 20 µL PBS with 2% BSA were added to Cel-Line (Thermo Scientific) IFA slides as described before [22]. Pre-immune control sera (I1-1) were added at a single dilution of 1:50; the post-immune samples of 52 days after the fourth injection (I(4)+52) were added at 2-fold dilutions starting at 1:50. Anti-PfCSP monoclonal antibody 2A10 [23] was used as positive control. After incubation at 37°C for 1 hour, slides were washed and Alexa fluor 488 conjugated goat anti-human IgG (Molecular Probes, cat# A11013) (1:250 in 0.2% Evans Blue) was added followed by incubation at 37°C for 1 hour and a washing step. Vectashield mounting medium (Vector Laboratories) was added to each well, and a cover slip placed on the slide. Samples were assessed with an Olympus BX51 fluorescence microscope at 400x magnification. The positive control was a serum specimen from a volunteer immunized with PfSPZ Vaccine (radiation attenuated PfSPZ) and a malaria naive serum sample was used as negative control [22]. The endpoint titer was defined as the last serum dilution at which fluorescence intensity was higher than pre-immune sera. A post-immunization serum sample was considered positive if it had fluorescence at a dilution of 1:50 or higher, and the pre-immunization serum from that volunteer was negative at 1:50.

Analysis of cellular immune responses by FACS

For the assessment of Pf-specific immune responses, in vitro re-stimulation assays on PBMCs were performed as described previously [24]. Briefly, cryopreserved PBMCs were thawed and stimulated in vitro for 24 hours with glycerol-cryopreserved schizont-stage Pf NF54 infected erythrocytes (PfRBCs) and purified Pf NF54 sporozoites in the presence of anti-human CD107a antibody (Biolegend, Pacific Blue, H4A3) at 100 μ L/well (final concentrations: 5×10^6 PBMC/mL; 10×10^6 PfRBC/well; 1.25×10^6 PfSPZ/mL). Uninfected red blood cells (uRBCs) and medium with 1% HSA (AlbuRx 25, ZLB Behring AG, Switzerland) were used as a negative control. For the last 4 hours, Brefeldin A (Sigma, 10 μ g/mL) and Monensin (Sigma, 2 μ M) were added, along with PMA (Sigma, 50 ng/mL) and ionomycin (Sigma, 1 μ g/mL) in positive control wells. Cells were stained with a viability marker (live/dead fixable dead cell stain aqua; Invitrogen) and antibodies against CD3 (PerCp, UCHT1), IFN- γ (PECy7, 4S.B3) and granzyme B (FITC, GB11; all Biolegend); CD4 (ECD, SFC12T4D11), γ δ T cell receptor (PE, IMMU510; both Beckman-Coulter), CD8 (BD Biosciences, APC-H7, SK1) and CD56 (eBioscience, biotin, MEM188 with eBioscience Streptavidin eFluor 660) [24]. Intracellular cytokine staining was performed using the Fixation and Permeabilization buffer kit (eBioscience). For every individual subject, all time-points were thawed, stimulated and stained within the same experimental round. Flow cytometry was performed on a 9-color Cyan ADP (Beckman Coulter) and data were analyzed using FlowJo software (version 9.6.4; Tree Star). Gating of cytokine-positive cells was performed automatically, based on the geometric Mean Fluorescent Intensity (MFI) of cytokine negative PBMCs for each subject, time-point and stimulus. Responses to uRBCs were subtracted from the response to PfRBCs for every subject on every time-point.

Analysis of chloroquine and monodesethylchloroquine concentrations

One hundred μ L of plasma of volunteers in groups 1 and 2 collected on the day before CHMI (C-1) was used to assess chloroquine and monodesethylchloroquine levels. The plasma samples were precipitated by methanol (400 μ L) containing hydroxychloroquine (50 ng/mL). After mixing and centrifugation, supernatants were diluted to half in ammonium formate solution 20 mmol/L with formic acid (0.5% v/v) and 15 μ L per sample was injected into the system. Chloroquine and monodesethylchloroquine were separated and quantified by liquid chromatography mass detection (TSQ Quantum Ultra, ThermoFisher, France) using an Atlantis DC18 (100 mm x 2.1 mm, 3 μ m) column (Waters, France) using water/methanol (95/5% v/v) with formic acid (0.1% v/v) as mobile phase. The flow rate was 0.30 mL/min and the column temperature was kept at 25°C. Hydroxychloroquine was used as internal standard. Data were acquired in the positive-ion mode with an electrospray (ESI) source. Multiple reaction monitoring (MRM) was used for data collection.

Statistical methods

Statistical analyses were performed with GraphPad Prism 5. The difference in AEs between 2 groups was calculated by unpaired Student *t* test on the cumulative duration of AEs. Differences between groups in pre-patent periods by qPCR, in pre-patency between thick smear and qPCR (Δ pre-patency), and in antibody levels were tested by the Mann-Whitney test. Differences in antibody concentrations between time points within a group were tested by Wilcoxon signed rank test.

Results

Trial overview

Thirty of 65 screened subjects (median age 21 years; range 18–27 years) were included in the study (Figure 1). Twenty subjects under chloroquine chemoprophylaxis (groups 1 and 3) were immunized by ID injection 3 times at monthly intervals with 75,000 cryopreserved PfSPZ, 10 controls (groups 2 and 4) received chloroquine chemoprophylaxis and ID injections of NS on the same schedule. Groups 1 and 2 underwent CHMI 60 days after the last immunization, corresponding to 33 days after the last chloroquine dose. Two subjects (20%) in vaccine group 1 remained thick smear negative through day 21 after CHMI (Table 1). One subject in vaccine group 3 who had tetanus vaccination after the third immunization session was excluded due to the safety procedures adopted after the first CHMI, because of the SAE. Another subject in group 3 was unable to continue participation for the fourth immunization due to logistical reasons. According to protocol, 13/15 subjects (8/10 in group 3; 5/5 in group 4) received a fourth immunization of 75,000 cryopreserved PfSPZ at 168 days after the third immunization (Figure 1). Five of these 13 subjects were not able to participate in the CHMI by mosquito bite for logistical reasons. Altogether 4 subjects in each group underwent CHMI at 109 days after the last chloroquine dose, corresponding to 137 days after their last injection of PfSPZ or NS.

Safety and tolerability during PfSPZ-CVac immunizations

Immunizations were well tolerated. There were no signs of local reactogenicity in vaccinees or controls. Eleven of 20 PfSPZ-CVac recipients (7 in group 1 and 4 in group 3) reported predominantly mild to moderate probably/possibly solicited AEs (mean duration, 0.3 ± 0.5 days) beginning on days 1 to 25 after the first 3 immunizations. Six of 10 NS recipients (3 in group 2 and 3 in group 4) reported predominantly mild to moderate probably/possibly solicited AEs (mean duration, 0.3 ± 0.4 days) beginning on days 1 to 20 after the first 3 immunizations. The other 9 PfSPZ-CVac and 4 NS inoculated volunteers did not report any complaints. There was no difference in the cumulative duration of probably/possibly solicited AEs per subject between vaccinees (groups 1 and 3) and controls (groups 2 and 4) ($P=0.52$). There were no solicited AEs after the fourth PfSPZ-CVac injection in vaccine group 3 and 1 mild headache in control group 4. Overall, the most commonly

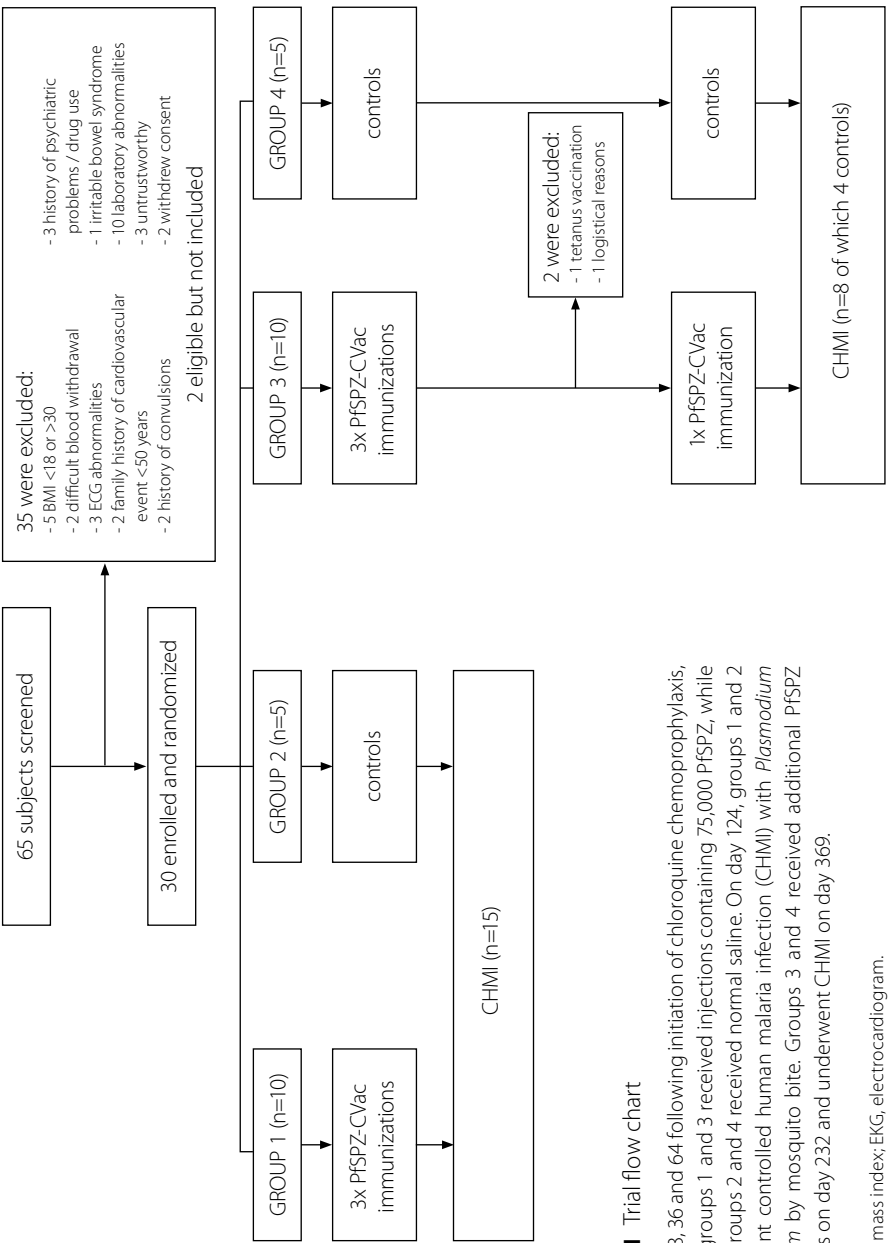


Figure 1 Trial flow chart

On days 8, 36 and 64 following initiation of chloroquine chemoprophylaxis, vaccine groups 1 and 3 received injections containing 75,000 PfSPZ, while control groups 2 and 4 received normal saline. On day 124, groups 1 and 2 underwent controlled human malaria infection (CHMI) with *Plasmodium falciparum* by mosquito bite. Groups 3 and 4 received additional PfSPZ injections on day 232 and underwent CHMI on day 369.

BMI, body mass index; EKG, electrocardiogram.

Table 1 Trial summary table

	Group 1	Group 2	Group 3	Group 4
	n=10	n=5	n=10 (n=4 received CHMI)	n=5 (n=4 received CHMI)
Dose (number of PfSPZ)	3 x 75,000	0	3 x 75,000 (n=2) 4 x 75,000 (n=8)	0
Route of administration	ID	ID	ID	ID
Number of volunteers who became TS+	8	5	N/A	N/A
Listing of times to TS+ (days)	10.5, 10.5, 11, 12, 12, 14, 14.5, 15	10.5, 10.5, 13.5, 14, 16	N/A	N/A
Geometric mean time to TS+ (days)	13.7	12.7	N/A	N/A
Listing of parasite density by qPCR at time of TS+ (parasites/ μ L blood)	14, 26, 13, 40, 46, 66, 24, 27	44, 120, 75, 32, 1	N/A	N/A
Geometric mean parasite density by qPCR at time of TS+ (parasites/ μ L blood)	27.9	26.3	N/A	N/A
Number of volunteers who became qPCR+ (%)	10 (100%)	5 (100%)	4 (100%)	4 (100%)
Listing of times to qPCR+ (days)	7.0, 7.0, 7.0, 7.0, 7.0, 7.0, 9.0, 10.5, 10.5, 10.5	7.0, 7.0, 7.0, 7.0, 10.5	7.0, 10.5, 10.5, 10.5	7.0, 7.0, 7.0, 10.0
Geometric mean time to qPCR+ (days)	8.1	7.6	9.5	7.7
Listing of parasite densities by qPCR at time of qPCR+ (parasites/ μ L blood)	0.08, 1.01, 0.09, 0.13, 0.25, 0.07, 0.08, 0.05, 0.05, 0.08	1.02, 0.47, 0.90, 0.13, 1.78	0.74, 1.40, 0.08, 0.06	0.86, 0.36, 0.97, 0.52
Geometric mean parasite density by PCR at time of qPCR+ (parasites/ μ L blood)	0.11	0.63	0.27	0.63

ID, intradermal; N/A, not applicable; TS+, thick smear positive; qPCR+, quantitative real-time polymerase chain reaction positive.

Table 2 Adverse events during immunizations #1–4

Any adverse event ^c	PfSPZ-CVAc groups 1 ^a (n=10) and 3 (n=2 ^a / n=8 ^b)			Control groups 2 ^a (n=5) and 4 ^b (n=5)		
	Number of volunteers	Mean duration ± sd (days)	Occurrence after injections (days)	Number of volunteers	Mean duration ± sd (days)	Occurrence after injections (days)
Abdominal pain	2	0.9 ± 1.0	-6, 25	2	0.1 ± 0.02	1–10
Chills	2	1.1 ± 1.2	2–4, 4	N/A ^d	N/A	N/A
Diarrhoea	1	0.6	20	N/A	N/A	N/A
Headache	6	0.2 ± 0.2	-2–33	3	0.5 ± 0.6	1–20
Nausea	4	0.4 ± 0.6	-6–27	1	0.1 ± 0.1	20
Vomiting	2	0.02 ± 0.0	-1, 6	N/A	N/A	N/A
Any	11	0.3 ± 0.5	N/A	6	0.3 ± 0.4	N/A

a. Total of 3 PfSPZ or normal saline immunizations.

b. Total of 4 PfSPZ or normal saline immunizations.

c. Subjects could have more than 1 adverse event. Only solicited adverse events that were possibly or probably related to the study are listed.

d. N/A, not applicable

reported AE was headache (6/20 vaccine subjects and 3/10 controls, Table 2), which occurred once as the single reported grade 3 adverse event.

After the first PfSPZ-CVac immunization, a remarkable unsolicited AE occurred in 1 subject in vaccine group 3. Several hours after the fourth chloroquine dose, transitory urticaria developed at multiple sites of the body lasting for 3 days (corresponding to days 5–8 after PfSPZ Challenge injections). The subject did not receive any treatment for the urticaria. This subject had a raised D-dimer level 2 days after resolution of the urticaria (>500 ng/mL; i.e. 1060 ng/mL) that decreased to 520 ng/mL within the next 4 days. The volunteer continued in the study, received 3 immunizations with PfSPZ-CVac and underwent CHMI, but did not develop urticaria or any other indication of an allergic reaction. However, the D-dimer levels were elevated after each of the 3 following immunizations (range 520–1350 ng/mL).

None of the 20 PfSPZ-CVac recipients developed parasitaemia, as retrospectively detected by qPCR, and all thick smears remained negative. Furthermore, lymphocyte and platelet counts did not decline after immunizations.

Protective efficacy after CHMI by *P. falciparum*-infected mosquitoes

All 5 controls in group 2 became thick smear positive. However, this group showed a wide variation in pre-patent periods (median 13.5 days, range 10.5–16 days; Figure 2) and a Δ pre-patency of 3.0–9.0 days, which was significantly longer compared to previous studies ($P=0.006$). This wide range is explained by the prolonged parasitaemia below the detection limit for microscopy in 2 of the control subjects (represented by the brown and pink lines in Figure 3). Retrospective parasitaemia measurement by qPCR revealed a

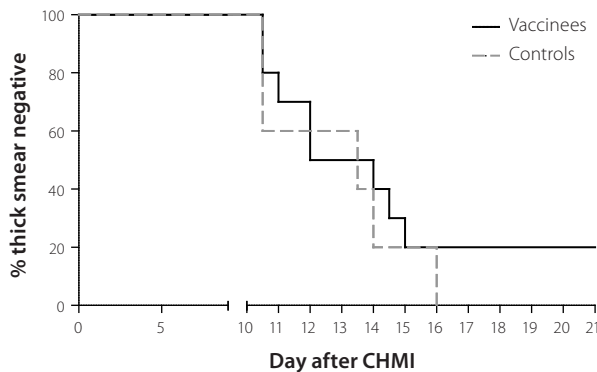


Figure 2 Time to thick smear positivity after controlled human malaria infection #1

The time to thick-smear positivity is shown for 10 PfSPZ-CVac recipients (group 1, black line) and 5 control subjects (group 2, grey dashed line).

CHMI, controlled human malaria infection.

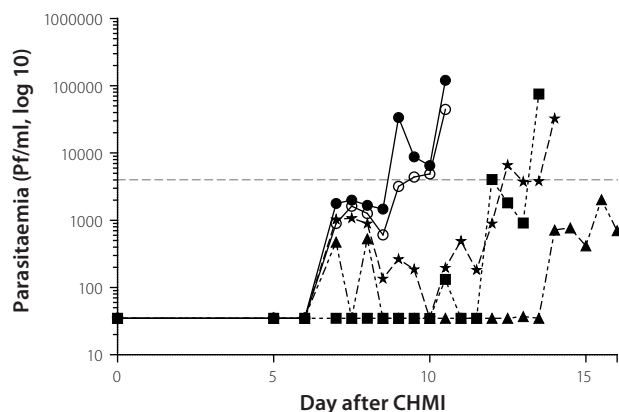


Figure 3 Parasite density after controlled human malaria infection #1

Individual parasite density curves of control subjects ($n=5$) measured by qPCR are shown up to day of treatment, based on diagnosis by thick smear. The grey dotted line indicates the average parasite detection limit for microscopy. Four of 5 subjects were first positive on day 7. One subject was first positive on day 10.5.

CHMI, controlled human malaria infection.

median pre-patent period of 7.0 days (range 7.0–10.5 days), comparable to previous studies [7,24] and (clinicaltrials.gov:) NCT01422954 ($P=0.56$).

Eight out of 10 vaccinees developed patent parasitaemia by thick smear (median 12 days, range 10.5–15 days), while two subjects in group 1 remained negative throughout the 21-day follow-up period (Figure 2). On the day before CHMI (C-1), plasma chloroquine was deemed to be below the minimum therapeutic concentration in vivo in all 15 subjects [25]. However, it is noteworthy that both protected subjects had higher levels of chloroquine in plasma (13 $\mu\text{g/L}$) than all of the non-protected subjects (≤ 5 $\mu\text{g/L}$). Their monodesethylchloroquine levels (principal metabolite of chloroquine) were not different than the other subjects (< 5 $\mu\text{g/L}$). Furthermore, both protected subjects showed positive qPCRs peaking on either day 7 post-CHMI (85 and 252 parasites/mL) or day 7.5 (265 parasites/mL). Finally, the median pre-patent period by qPCR of group 1 was 7 days (range 7.0–10.5 days) and similar to control group 2 ($P=0.55$). The combined data suggest that a parasite killing effect of residual chloroquine levels cannot be excluded in the 2 protected individuals. The second CHMI was administered to groups 3 and 4 after a fourth immunization. All 8 subjects became qPCR positive (Figure 4) with a median pre-patent period of 10.5 days (range 7–10.5 days) in immunized volunteers ($n=4$) and 7 days (range 7–10 days) in controls ($n=4$), which was not statistically different ($P=0.11$).

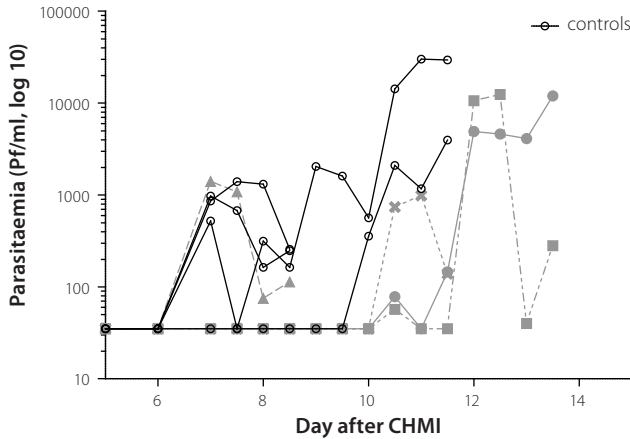


Figure 4 Parasite density after controlled human malaria infection #2

Parasite densities are shown until the day of treatment, based on diagnosis by qPCR. Each line represents a single subject; the grey lines represent subjects of vaccine group 3 ($n=4$), the black lines subjects of control group 4 ($n=4$).

CHMI, controlled human malaria infection.

Adverse events after CHMI by *P. falciparum*-infected mosquitoes

All subjects in groups 1 and 2 experienced solicited AEs possibly or probably related to CHMI (mean number of AEs per subject in group 1: 6.9, mean duration 0.6 ± 1.2 days; group 2: 8.4, mean duration 0.6 ± 0.7 days), with headache ($n=29$), fever ($n=20$) and nausea ($n=19$) most commonly reported (Table 3). There was no significant difference between the cumulative duration of AEs per subject in group 1 compared to group 2 (3.8 versus 4.7 days, respectively; $P=0.64$). One SAE occurred in a subject in vaccine group 1 on day 12 after CHMI and 2 days after initiation of treatment with atovaquone/proguanil (72 days after last PfSPZ-CVac immunization), which was diagnosed as acute myocarditis [16]. Abnormal laboratory values normalized without complications in all subjects.

All volunteers in group 3, except for 1, experienced solicited AEs possibly or probably related to CHMI (mean number of AEs per subject in group 3: 4.8, mean duration 0.5 ± 0.6 days; in group 4: 6.8, mean duration 0.9 ± 1.9 days), with headache ($n=15$), nausea ($n=9$) and chills ($n=7$) as most common symptoms (Table 3). The cumulative duration of AEs per subject in group 3 was similar to group 4 (2.3 versus 6.0 days, respectively; $P=0.051$). Additionally, there was no significant difference between control groups 2 and 4 ($P=0.51$), although there was a trend towards a lower frequency of AEs in group 4 in which antimalarial treatment was initiated after qPCR instead of thick smear positivity.

Table 3 Adverse events after mosquito CHMI

CHMI #1	PfSPZ-CVac group 1 (n=10)		Control group 2 (n=5)	
Any adverse event ^a	Number of volunteers	Mean duration \pm sd (days)	Number of volunteers	Mean duration \pm sd (days)
Abdominal pain	2	0.5 \pm 0.1	2	0.1 \pm 0.1
Chest pain, unspecified	1	0.0	0	N/A ^b
Chills	6	0.2 \pm 0.1	3	0.4 \pm 0.2
Diarrhoea	0	N/A	1	0.1 \pm 0.0
Dizziness	1	1.2	2	0.1 \pm 0.0
Fatigue	2	4.0 \pm 5.2	1	0.4 \pm 0.3
Fever	7	0.3 \pm 0.3	4	0.6 \pm 0.4
Headache	8	0.5 \pm 0.4	5	0.9 \pm 1.1
Malaise	1	0.3	1	0.5
Myalgia	2	1.2 \pm 0.7	3	1.2 \pm 0.7
Nausea	5	0.3 \pm 0.3	3	0.3 \pm 0.4
Vomiting	2	0.0 \pm 0.0	1	0.2
Any	10	0.6 \pm 1.2	5	0.6 \pm 0.7
Grade 3 adverse event				
Fever	3	0.2 \pm 0.1	1	0.5
Headache	1	0.4	0	N/A
Nausea	0	N/A	1	0.3
Vomiting	2	0.0 \pm 0.0	1	0.2
Any	4	0.1 \pm 0.2	2	0.3 \pm 0.2

CHMI #2	PfSPZ-CVac group 3 (n=4)		Control group 4 (n=4)	
Any adverse event	No. of volunteers	Mean duration \pm SD (days)	No. of volunteers	Mean duration \pm SD (days)
Abdominal pain	0	N/A	2	0.3 \pm 0.2
Chills	1	0.3	2	0.0 \pm 0.0
Dizziness	0	N/A	1	0.1
Fever	1	0.6	2	0.8 \pm 1.0
Headache	3	0.4 \pm 0.5	3	2.4 \pm 3.2
Malaise	1	2.2	0	N/A
Myalgia	1	0.6 \pm 0.6	1	1.5
Nausea	3	0.3 \pm 0.2	2	0.2 \pm 0.1

Table 3 Continued

CHMI #2	PfSPZ-CVac group 3 (n=4)		Control group 4 (n=4)	
Any adverse event	No. of volunteers	Mean duration \pm SD (days)	No. of volunteers	Mean duration \pm SD (days)
Vomiting	1	0.0	0	N/A
Any	3	0.5 \pm 0.6	4	0.9 \pm 1.9
Grade 3 adverse event				
Fever	0	N/A	1	0.4
Headache	0	N/A	1	0.1
Vomiting	1	0.0	0	N/A
Any	1	0.0	1	0.3 \pm 0.2

a. Subjects could have more than 1 adverse event. Only solicited adverse events that were possibly or probably related to the study are listed.

b. N/A, not applicable.

Humoral and cellular immune responses

First we addressed whether volunteers immunized with PfSPZ-CVac in group 1 developed specific antibodies to PfCSP, PflSA-1, or PfMSP-1 at 59 days after the third immunization and 1 day before CHMI. Five out of 10 subjects showed a greater than 2-fold rise of anti-PfCSP antibody titers compared to pre-immunization ($P=0.03$) ranging from 0.9 to 5.7 AU (median, 2.66). Antibodies to PflSA-1 and PfMSP-1 increased non-significantly in 3 and 2 subjects, respectively (range fold-increase: anti-PflSA-1, 0.75–1.23; anti-PfMSP-1, 0.52–1.15; Figure 5a). Anti-PfCSP antibody titers were comparable in both PfSPZ-CVac groups (1 and 3) before the third immunization ($P=0.41$; Figure 5b).

Next, we investigated the boosting effect of the third and fourth immunization, respectively. The third immunization increased anti-PfCSP antibodies in 7/10 subjects in group 1 and in only 1 subject more than 2-fold (I3-1 versus I3+59; median fold increase with range: 1.26 [0.79–2.45]; $P=0.28$). In contrast, anti-PfCSP antibodies were boosted in all 8 subjects of group 3 and by at least 2-fold in 3 subjects (I4-1 versus I4+52; median fold increase with range: 1.43 [1.25–5.74]; $P=0.008$; Figure 5c). Similarly, 7/8 subjects of group 3 developed antibodies against PfSPZ by immunofluorescence assay (IFA) after the fourth immunization (anti-PfSPZ titers ranged from 50 to 400) in contrast to controls who did not develop antibodies (anti-PfSPZ titer <50). While in group 3 the absolute magnitude of antibodies to PfCSP was not greater after the fourth dose than in group 1 after the third dose ($P=0.24$), the proportion of responders increased with a fourth dose. These data suggest that 4 immunizations increase the number of responders but that absolute

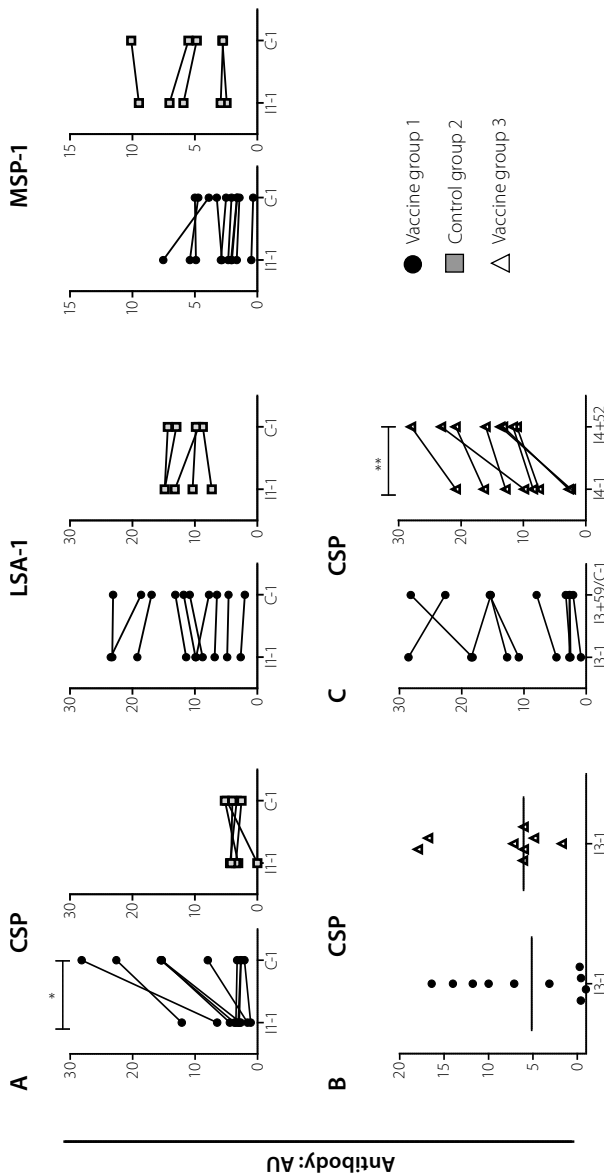


Figure 5 Specific antibody responses induced by immunization by PfSPZ-CVac

Parasite-specific plasma antibody responses are shown for vaccine group 1 (n=10, black circles), control group 2 (n=5, grey squares) and vaccine group 3 (n=8, white triangles) at the following time-points: (A) 1 day before the first immunization (I1-1) and 59 days after the third immunization (C-1); (B) 1 day before the third immunization (I3-1) with antibody-responses corrected for the subjects own background level; (C) 1 day before (I3-1) and 59 days after the third immunization (I3+59) (C-1) as well as 1 day before the fourth immunization (I4-1) and 52 days later (I4+52). Antibody responses are expressed as arbitrary units (AU) in relation to Tanzanian pooled serum (100). Each line represents a single subject. Differences were analysed using Wilcoxon matched-pairs signed rank test. Significant differences are indicated by asterisk with * ($P<0.05$), and ** ($P<0.01$).

AU, arbitrary unit; CSP, circumsporozoite protein; LSA-1, liver stage antigen 1; MSP-1, merozoite surface protein 1.

antibody levels did not increase. Antibodies against PfLSA-1 and PfMSP-1 were not significantly increased after the fourth immunization (data not shown, $P=0.64$ for anti-PfLSA-1 and $P=0.38$ for anti-PfMSP-1).

In contrast to humoral responses, neither IFN- γ , CD107a, nor granzyme B re-call responses to PfrBC or PfSPZ, which were found to be indicative of parasite exposure previously [24,26], were induced after 3 PfSPZ-CVac immunizations in any of the T-cell subsets analyzed (vaccine group 1 versus control group 2, data not shown). Furthermore, even after the fourth PfSPZ-CVac immunization administered in group 3 there were still no measurable responses to PfrBC when compared to control group 4 or pre-immunization (data not shown).

Discussion

This first clinical study of the PfSPZ-CVac approach to immunizing healthy malaria-naïve volunteers showed that the ID immunization regimen of 3 doses of 7.5×10^4 PfSPZ administered at 4-week intervals to subjects taking weekly chloroquine chemoprophylaxis was safe and well-tolerated, but did not confer protection against a homologous CHMI. In contrast, the 3-dose CPS-protocol using PfSPZ-infected mosquitoes for immunizations under weekly chloroquine cover at the same 4 week intervals, has induced >90% protection in a number of clinical trials [5,7].

Protective efficacy after immunization by CPS is almost certainly due to Pf parasites moving from the skin into the vascular system to the liver and then invading and fully developing in hepatocytes. In the CPS studies transient qPCR-detected blood-stage parasitaemia in particular after the first immunization was observed in the vast majority of volunteers. This reflects complete liver maturation followed by rapid chloroquine-mediated killing of blood-stage parasites. Under these conditions the host's immune system is exposed to a broad array of antigens for induction of protective pre-erythrocytic immune responses [21,27]. In a previous CPS trial dose-dependent sterile protection has been observed: 4/5 volunteers were protected after immunization with bites from 3 times 15, 8/9 volunteers by 3 times 10 and 5/10 volunteers by 3 times 5 infected mosquitoes respectively [24].

In this first study of the PfSPZ-CVac approach, we believe that inadequate numbers of PfSPZ made the journey from skin through the vascular system into the liver for full development. This hypothesis was supported by the lack of protection and the weak humoral and absent cellular immune responses against Pf antigens. CPS-induced humoral responses have been shown to correlate with the degree of antigen exposure [21]. While anti-PfCSP antibody responses after PfSPZ-CVac immunizations were comparable to after CPS immunizations [21], indicating exposure to adequate numbers of PfSPZ, anti-LSA-1 and anti-MSP-1 antibody responses were poor and absent respectively, reflecting very limited hepatocyte invasion and liver-stage development. This interpretation was further supported by the absence of cellular re-call responses after PfSPZ-CVac immunizations i.e.

parasite-specific IFN- γ , CD107a, or granzyme B responses, indicative of parasite exposure [24,26] and degranulation of CD4 T cells, associated with protection [24].

In contrast to CPS-immunization by PfSPZ-infected mosquito bites, PfSPZ-CVac was administered ID by needle injection. Murine data have shown that the route of administration of fresh and cryopreserved PfSPZ is a key determinant of successful liver infection; IV and IM injections result in significantly higher (~50 fold and 2- to 3-fold, respectively) liver loads compared to ID and SC injections [28]. Furthermore, Ploemen et al. demonstrated that both IM and ID routes increased liver loads when using smaller volumes and injections at multiple sites. This method approaches the way anopheline mosquitoes successfully administer sporozoites: in some cases before a capillary is found the female mosquito probes the dermal skin several times, while injecting tiny bits of sporozoite-containing saliva [29]. Increased parasite liver loads in mice after IV inoculation of both radiation-attenuated sporozoites and sporozoites in combination with chloroquine treatment were associated with augmented cellular immune responses and higher protective efficacy after challenge compared to ID inoculation [30].

Clinical trials of PfSPZ Challenge have also demonstrated that IV administration is more efficient than IM, which is more efficient than ID administration. The goals of these trials have been to identify the lowest dose of PfSPZ Challenge that gives 100% infection rates with a pre-patent period by thick blood smear of 11 to 11.5 days, and a decrease of pre-patent period with increasing dose of PfSPZ (e.g. a dose response). One hundred percent infection rates have been achieved by IV, direct venous inoculation (DVI), IM, and ID administration (Mordmueller, submitted). However, a pre-patent period of <11.5 days and a dose response have only been achieved by IV/DVI and IM administration (personal communication by SL Hoffman). It takes 23–25 times as many PfSPZ administered IM (7.5×10^4) as IV (3.2×10^3) to achieve a pre-patent period of <11.5 days (Stephen Hoffman, personal communication). Thus far this pre-patent period has not been achieved by ID administration. This difference between IV and ID (or SC) administration has also been seen with radiation attenuated, aseptic, purified, cryopreserved PfSPZ, PfSPZ Vaccine. Four to 6 doses of 1.35×10^5 PfSPZ of PfSPZ Vaccine SC or ID gave minimal immune responses and no protection [22], whereas 5 doses of 1.35×10^5 PfSPZ IV gave excellent immune responses and 100% protection [31].

The current dose of 7.5×10^4 PfSPZ-CVac administered ID was well tolerated with remarkably few and mild adverse events similar to controls receiving only NS. Once the methodology of PfSPZ-CVac administration will be optimized and higher liver loads and transient parasitaemia are present, it is to be expected that the frequency and severity of AEs will likely match previous CPS trials [5]. However, there was a cardiac SAE 59 days after the last dose of PfSPZ Challenge, 12 days after CHMI, and 2 days after initiation of treatment with Malarone for a positive blood slide in a subject of vaccine group 1. The subject was diagnosed as having acute myocarditis [16], but the pathophysiological basis for this acute myocarditis remains unclear and a definitive aetiology could not be established [16].

Interestingly, there was a discrepancy in the protection against CHMI by mosquito bite as assessed by thick smear and retrospective qPCR in group 1. Two of the 10 subjects were thick smear negative but positive by qPCR on days 7 and 7.5 post-CHMI in the presence of a ~2.5-fold higher chloroquine (13 µg/L) plasma concentration on day 1 before CHMI compared to non-protected subjects (≤ 5 µg/L). This raises the possibility that the early parasitaemias in these 2 volunteers could have been cleared by residual chloroquine. Another finding suggesting an impact of chloroquine was the significantly prolonged Δ pre-patency in control group 2 (Figure 3) compared to controls of recent previous CHMI studies. However, it is noteworthy that in a previous CHMI trial where similar plasma concentrations were measured (range <5 to 14 µg/L for chloroquine and <5 µg/L for monodesethylchloroquine) [5], all 5 controls became thick-smear positive between days 7 and 11 after CHMI. These plasma concentrations were deemed to be below the minimum therapeutic concentration in vivo based on literature [Rombo et al. 1987: min. = 30 µg/L], but also because identical blood-stage parasite multiplication kinetics were seen in control subjects compared to previous studies suggesting that any residual chloroquine levels had no measurable parasitocidal effect [32,33].

In conclusion, this study shows that the dose and ID immunization regimen of PfSPZ-CVac used in this trial was safe, but did not provide protection against a homologous CHMI by mosquito bite. The lack of protection was almost certainly due to sub-optimal parasite exposure, as supported by weak humoral responses and the lack of T-cell responses previously shown to be associated with protection. In the next clinical trial PfSPZ Challenge will be administered by DVI in order to achieve consistent, high-level liver stage infection.

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CHAPTER 8

IDIOPATHIC ACUTE MYOCARDITIS DURING TREATMENT FOR CONTROLLED HUMAN MALARIA INFECTION: A CASE REPORT

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Abstract

A 23-year-old healthy male volunteer took part in a clinical trial in which the volunteer took chloroquine chemoprophylaxis and received 3 intradermal doses at 4-week intervals of aseptic, purified *Plasmodium falciparum* sporozoites to induce protective immunity against malaria. Fifty-nine days after the last administration of sporozoites and 32 days after the last dose of chloroquine the volunteer underwent controlled human malaria infection (CHMI) by the bites of 5 *P. falciparum*-infected mosquitoes. Eleven days post-CHMI a thick blood smear was positive (6 *P. falciparum*/μL blood) and treatment was initiated with atovaquone/proguanil (Malarone®). On the second day of treatment, day 12 post-CHMI, troponin T, a marker for cardiac tissue damage, began to rise above normal, and reached a maximum of 1,115 ng/L (upper range of normal = 14 ng/L) on day 16 post-CHMI. The volunteer had one ~20 minute episode of retrosternal chest pain and heavy feeling in his left arm on day 14 post-CHMI. ECG at the time revealed minor repolarization disturbances, and cardiac MRI demonstrated focal areas of subepicardial and midwall delayed enhancement of the left ventricle with some oedema and hypokinesia. A diagnosis of myocarditis was made. Troponin T levels were normal within 16 days and the volunteer recovered without clinical sequelae. Follow-up cardiac MRI at almost 5 months showed normal function of both ventricles and disappearance of oedema. Delayed enhancement of subepicardial and midwall regions decreased, but was still present. With the exception of a throat swab that was positive for rhinovirus on day 14 post-CHMI, no other tests for potential aetiologies of the myocarditis were positive. A number of possible aetiological factors may explain or have contributed to this case of myocarditis including, i) *P. falciparum* infection, ii) rhinovirus infection, iii) unidentified pathogens, iv) hyper-immunization (the volunteer received 6 travel vaccines between the last immunization and the CHMI), v) atovaquone/proguanil treatment, or vi) a combination of these factors. Definitive aetiology and pathophysiological mechanism for the myocarditis have not been established.

Introduction

Controlled human malaria infections (CHMIs) have been used for nearly a century for treatment of neurosyphilis and for assessing interventions like drugs and vaccines for treating and preventing malaria. However, the modern era of CHMIs began in the mid 1980s, when laboratory reared *Anopheles* sp. mosquitoes were infected by feeding on cultures of *Plasmodium falciparum*-infected blood [1]. During the past 3 decades CHMI has been shown safe, well-tolerated and useful in evaluation of potential new antimalarial drugs and vaccines [2]. After exposure to bites of laboratory-reared mosquitoes infected with *P. falciparum* sporozoites (PfSPZ) clinical symptoms and signs of malaria are generally mild to moderate and last for a few days. The most commonly reported symptoms are headache, fever, myalgia and fatigue, and common laboratory abnormalities include clinically insignificant thrombocytopaenia and leukopaenia [3,4]. Subjects, who undergo CHMI, are closely monitored and immediately treated with antimalarials upon detection of parasitaemia. Due to frequent and intense clinical monitoring, initiation of treatment almost always occurs at parasite densities of less than 0.001% and often at 0.0001% [5], a density which is more than 1,000-fold lower than parasite densities associated with causing severe malaria.

Immunization of volunteers taking chloroquine chemoprophylaxis with whole PfSPZ administered by mosquito bites resulted in complete and long-lasting protection against CHMI with *P. falciparum*-infected mosquitoes [6,7]. This immunization approach is called ChemoProphylaxis with Sporozoites (CPS). Since CPS depends on inoculation of PfSPZ by mosquito bites, it cannot be an implementable vaccine. Recently, subjects were infected by needle and syringe inoculation of aseptic, purified, cryopreserved PfSPZ, a product called PfSPZ Challenge [8,9]. Subsequently, a clinical trial was initiated in which volunteers taking chloroquine chemoprophylaxis were injected intradermally (ID) at 4-week interval with PfSPZ Challenge, an approach called the PfSPZ-CVac approach (=PfSPZ Chemoprophylaxis Vaccine), and then underwent CHMI.

Here, a very probable case of acute myocarditis is described in a volunteer who had taken chloroquine chemoprophylaxis, was inoculated 3 times at 4-week intervals with PfSPZ Challenge, received 6 travel-related routine vaccines after this immunization procedure, had CHMI by the bites of 5 PfSPZ-infected mosquitoes 8.5 weeks after the last dose of PfSPZ Challenge and 4.5 weeks after the last dose of chloroquine, had a sore throat on day 9 after CHMI, developed *P. falciparum* parasitaemia that was treated 11 days after CHMI, and had asymptomatic initial elevation of troponin T levels 12 days after CHMI.

Case presentation

A 23-year-old healthy male volunteer was enrolled in a double-blind, placebo-controlled trial that assessed the safety, tolerability, and protective efficacy against CHMI by PfSPZ-infected mosquitoes of ID administration of aseptic purified cryopreserved PfSPZ (PfSPZ Challenge) in volunteers taking weekly 300 mg chloroquine prophylaxis, the PfSPZ-CVac approach. His medical history was unremarkable, and he did not smoke or use illicit drugs. His mother had a history of hypertension and his paternal grandfather had a history of heart valve defects and a possible myocardial infarction at the age of 70. At inclusion, physical examination was within normal limits with a blood pressure of 139/76 mmHg, heart rate of 55 beats per minute and a body mass index of 20.2 kg/m². Electrocardiography (ECG) showed a commonly seen normal variant of incomplete right bundle branch block (Supporting Information [SI] File 1). Standard laboratory tests at inclusion were normal (Table 1).

From October to December 2012, he received 3 ID injections at 4-week intervals of 7.5×10^4 PfSPZ of PfSPZ Challenge (Pf NF54) diluted in phosphate buffered saline with 1% human serum albumin. From day 3 until day 8 after the first immunization he reported a sore throat and symptoms of a common cold (i.e., stuffy nose and coughing) with mild chills for a few hours. No complaints were reported after the second and third immunizations. No clinically significant laboratory abnormalities were found during the immunization period.

Fifty-nine days after the third and last immunization and 32 days after his last dose of chloroquine, he underwent CHMI by the bites of 5 *P. falciparum*-infected mosquitoes (Pf NF54). On day 9 post-CHMI he complained of a sore throat. On day 11 post-CHMI his thick blood smear became positive (6 *P. falciparum*/μL blood; 0.00012% infected erythrocytes) and standard treatment with Malarone® (1,000 mg atovaquone plus 400 mg proguanil once daily for 3 days) was initiated. Retrospective assessment of parasitaemia by quantitative real-time polymerase chain reaction (qPCR) revealed 13,293 parasites/mL (0.00026% infected erythrocytes) on day of thick smear positivity. On that day he complained of minor chills and headache for a few hours with a highest recorded sublingual temperature of 37.5°C. Platelet and lymphocyte counts decreased to $97 \times 10^9/L$ (normal range = $141\text{--}400 \times 10^9/L$) and $0.60 \times 10^9/L$ (normal range = $1.0\text{--}3.5 \times 10^9/L$), respectively, as often seen in malaria positive individuals [5,10]. The level of troponin T by a highly sensitive assay was normal (i.e., 8 ng/L; upper limit of normal = 14 ng/L). Troponin T is a specific marker for myocardial tissue damage.

On the second day of Malarone treatment (day 12 post-CHMI) the troponin T level was elevated at 45 ng/L and increased to 63 ng/L in the evening. No abnormalities were seen on ECG. Apart from mild headache and fatigue on the following day (day 13 post-CHMI) the volunteer was asymptomatic, but the troponin T was 197 ng/L in the morning and 299 ng/L in the afternoon. The blood pressure was 114/60 mmHg and the ECG revealed

Table 1 Laboratory findings

Haematology and biochemistry tests	Normal range	Inclusion	C+11	C+12	C+13	C+14	C+15	C+16	C+17	C+20	C+28
Haemoglobin (mmol/L)	8.5–10.8	10.1	9.4	10.2	9.2	8.2	8.2	8.2	8.0	9.5	9.6
Haematocrit (L/L)	0.41–0.53	0.47	0.43	0.47	0.43	0.38	0.37	0.38	0.37	0.44	0.44
Leukocytes ($\times 10^9/L$)	3.6–10.7	4.9	4.2	3.3	3.2	3.1	3.9	4.0	4.8	7.7	5.4
Neutrophils ($\times 10^9/L$)	2.0–7.5	2.16	3.04	1.85	1.77	1.26					2.12
Lymphocytes ($\times 10^9/L$)	1.0–3.5	2.05	0.60	0.79	0.88	1.13					2.57
Monocytes ($\times 10^9/L$)	0.3–1.0	0.43	0.48	0.52	0.47	0.55					0.47
Eosinophils ($\times 10^9/L$)	≤ 0.64	0.21	0.08	0.08	0.10	0.09					0.21
Basophils ($\times 10^9/L$)	< 0.10	0.03	0.03	0.02	0.02	0.03					0.05
Thrombocytes ($\times 10^9/L$)	141–400	138	97	94	84	89	108	123	129	200	162
Sodium (mmol/L)	135–143	139			138					142	
Potassium (mmol/L)	3.7–5.0	3.8			4.0					3.8	
Creatinine ($\mu\text{mol/L}$)	60–132	87			82	74	83	80	76	75	
Urea nitrogen (mmol/L)	2.5–8.1	5.1			4.6					5.8	
Alkaline phosphatase (U/L)	≤ 126	66			82	84	76	77	76		
Aspartate aminotransferase (U/L)	≤ 38	16			49	81	66	41	32		
Alanine aminotransferase (U/L)	≤ 49	23			33	42	37	41	40		
Lactate dehydrogenase (U/L)	≤ 250	110	142	181	222	316	262	258	222		154
γ Glutamyl-transferase (U/L)	≤ 55	22			18	21	19	23	22		
Creatine Kinase (U/L)	≤ 170			175	284	501	320	133	70		
Troponin T (ng/L)	≤ 14	6	8	45	197	596	829	1115	675	18	7
NT-proBNP (pg/mL)	< 88					197			128	67	
CRP (mg/L)	≤ 10				22	16	11	8	< 5	< 5	
D-dimer (ng/mL)	≤ 500	< 500	< 500	570	< 500	< 500	< 500	< 500	< 500		< 500

Clinical laboratory findings at inclusion (day before the start of the trial), on day of thick smear positivity (C+1), day 11 after CHMI) and subsequent days 12, 13, 14, 15, 16, 17, 20, and 28 after CHMI.

mild repolarization disturbances with diffuse ST-T-segment elevation, suggestive of pericarditis (SI File 2). The echocardiogram showed mild hypokinesia of the inferior wall and a slightly diminished global left ventricle (LV)-function (calculated LV ejection fraction of 53%; normal range for a young man is >55%). Malarone treatment was completed and qPCR for *P. falciparum* was negative on day 13 post-CHMI. Although the subject did not have any cardiac or chest symptoms, he was hospitalized at the cardiology department according to safety protocol for telemetric ECG-monitoring and follow-up of troponin T levels.

That night at ~01:00 AM he experienced retrosternal pain and a heavy feeling in his left arm. After approximately 10 minutes sublingual nitroglycerin spray was administered; the chest pain did not disappear immediately, but only 10 minutes after administration of nitroglycerin. The pain was not related to breathing and there were no concomitant complaints or signs of dyspnoea, pyrosis or ructus. The subject never had another episode of chest pain. Cardiac MRI several hours later on day 14 post-CHMI showed: i) slightly increased T2-weighted signal intensity in the basal- and mid-inferolateral and partly in the mid-anterolateral myocardial segments, matching minor oedema (Figure 1a); ii) focal areas of subepicardial and midwall delayed enhancement in the basal- and mid-inferior and basal- and mid-inferolateral segments after administration of gadolinium contrast (Figure 1b and c); iii) hypokinesia basal- and mid-inferior and mild hypokinesia basal- and mid-inferolateral. These findings were interpreted as indicative of myocarditis. Treatment was started on day 14 post-CHMI with a beta-blocker, metoprolol (25 mg twice daily), to reduce the chance of cardiac arrhythmia and according to the treatment guidelines for patients with reduced LV-function.

Troponin T levels continued to rise with a peak of 1,115 ng/L on day 16 after CHMI. The following days troponin T decreased and eventually returned to normal 16 days after initial increase, corresponding to 28 days post-CHMI. Creatine kinase (CK) showed a similar pattern of rising and falling, but returned to normal on day 16 post-CHMI. A biochemical marker of cardiac wall stress, N-terminal pro-hormone brain natriuretic peptide (NT-proBNP), was slightly elevated on days 15 and 17 post-CHMI, but was normal on day 20 post-CHMI (Table 1). A limited rise and fall of aspartate aminotransferase (AST) and lactate dehydro-genase (LDH) were found. The nonspecific marker for increased coagulation and inflammation, D-dimer, remained within the normal range and was only minimally elevated on the first day after thick smear positivity. Similarly, the inflammatory acute-phase protein, C-reactive protein (CRP) was only slightly elevated on days 13, 14, and 15 after CHMI (Table 1). Four days after admission (day 17 post-CHMI) ECG and echocardiogram had normalized (SI File 3; calculated LV ejection fraction was 62%; normal range is >55%) and the volunteer was discharged.

Apart from the single short episode of chest pain and a longer period of fatigue with occasional mild headache during and shortly after hospitalization, no other complaints were reported. The fatigue diminished after halving the dose of metoprolol to 25 mg

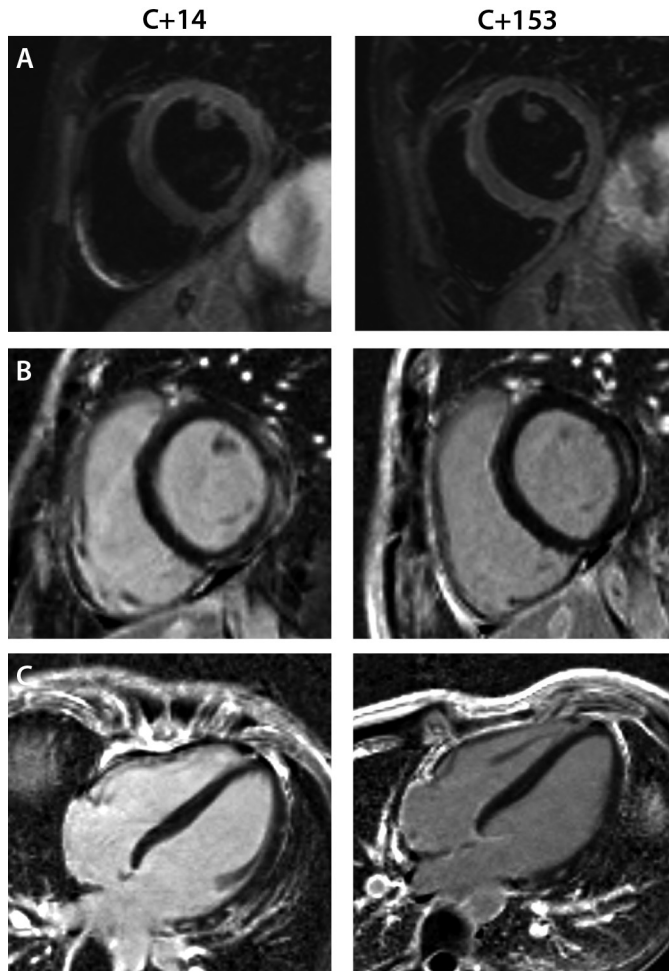


Figure 1 Cardiac MRI on day 14 and 153 after controlled human malaria infection

(A) Slightly increased T2-weighted signal intensity was observed in the basal-inferolateral segment of the left ventricle on day 14 after CHMI (C+14), which had disappeared on day 153 after CHMI (C+153); visualized on the short-axis dark blood STIR (short inversion time inversion recovery) recordings. (B and C) After administration of 15 mL gadolinium contrast subepicardial and midwall delayed enhancement was observed in the basal-inferolateral and basal-inferior segments of the left ventricle on day C+14, which had decreased on day C+153; visualized on the short-axis (B) and the 4-chamber (C) PSIR (phase sensitive inversion recovery) recordings.

once daily on day 23 post-CHMI. The remaining mild fatigue completely disappeared 3 weeks later on day 44 post-CHMI. After discharge the volunteer did not complain about occasional mild headache anymore.

The volunteer received pre-travel vaccines for diphtheria, tetanus, polio, typhus, hepatitis A and hepatitis B 14 days after the third injection of PfSPZ Challenge (46 days before CHMI). He had booster vaccinations for hepatitis A and B 40 days after the third injection (20 days before CHMI).

Polymerase chain reaction (PCR) analyses of throat smear, faeces and whole blood were carried out for viruses and bacteria known to cause myocarditis (Table 2). Throat smear PCR was positive for rhinovirus; all other PCR results were negative (Table 2). Virological, bacteriological and parasitological serology was performed on samples obtained 3 weeks before inclusion and on day 17 post-CHMI (Table 3) with repeat testing 18 days later (day 35 post-CHMI and 23 days after first elevation of troponin T). All serologic results were negative. Furthermore, urine toxicology screening for amphetamine-derivatives, cocaine, cannabinoids, diazepam, methadone, tramadol hydrochloride, and opiates was negative on day 17 after CHMI.

Table 2 PCR results for known infectious pathogens of myocarditis

	C+14	C+15	C+17
Throat smear tested by PCR for:			
Adenovirus, Bocavirus, Coronavirus, Chlamydia Psittaci, Enteroviruses, Metapneumovirus, Mycoplasma, Parechovirus, Parainfluenza 1–4, Rhinovirus, Respiratory Syncytial Virus, and Influenza A and B	positive for Rhinovirus		
Faeces tested by PCR for:			
Adenovirus (Adenotype 40 and 41), Astrovirus, Bocavirus, Enteroviruses, Norovirus, Parechovirus, Rotavirus, and Sapovirus		negative	
Blood tested by PCR for:			
Varicella Zoster Virus, Parvovirus, Epstein-Barr Virus, Cytomegalovirus, Q fever, and HIV load			negative

PCR results for known virological and bacteriological causes of myocarditis based on samples taken on day 14, 15, and 17 after CHMI (C+14, C+15, and C+17, respectively).

At almost 5 months after the first MRI, repeat cardiac MRI demonstrated good function of the left ventricle (calculated LV ejection fraction of 67%) with persistence of mild hypokinesia in the mid-inferior and mid-inferolateral segments of the left ventricle. The oedema had

Table 3 Serology results for known infectious pathogens of myocarditis

Sera tested for antibodies to:	Screening visit (paired with C+17)	C+17	C+17 (paired with C+35)	C+35
Echovirus pool (Types 4, 6, 9, 14, 24, and 30)	20	< 10 (negative)	< 10 (negative)	10
Coxsackie virus pool (Types A9, B1–B6)	20	10	10	20
Poliovirus	20	10	20	40
Adenovirus Ig	20	< 10 (negative)	20	20
Parvovirus IgM		negative		negative
Parvovirus IgG		64		64
Mycoplasma pneumoniae IgM	negative	negative		negative
Mycoplasma pneumoniae IgA	negative	negative		negative
Mycoplasma pneumoniae Ig	< 10 (negative)	< 10 (negative)	< 10 (negative)	< 10 (negative)
Chlamydia including Psittacosis Ig	< 10 (negative)	< 10 (negative)	< 10 (negative)	< 10 (negative)
Q fever phase 2 IgM	negative	negative		negative
Q fever Ig	< 10 (negative)	< 10 (negative)	< 10 (negative)	< 10 (negative)
Hepatitis B s-antigen		negative		negative
Hepatitis B anti-HBc		negative		negative
Hepatitis C Ig		negative		negative
Borrelia blot IgM		negative		negative
Borrelia blot IgG		negative		negative
Legionella serotype 1–7 IgM	negative	negative		negative
Legionella serotype 1–7 IgG	negative	negative		negative
Syphilis IgG		negative		negative
Toxoplasma IgG		negative		negative

Serology results for known virological, bacteriological and parasitological causes of myocarditis based on samples taken at the screening visit, on day 17, and 35 after CHMI (C+17 and C+35, respectively). Paired serologic analysis was performed with serum drawn at the screening visit (3 weeks before the start of the trial) for a number of pathogens. To detect potential delayed immune responses serologic analysis was repeated on day C+35. Again, paired serologic analysis was performed with serum drawn on day C+17 for a number of pathogens.

disappeared completely (Figure 1a) and concomitantly the delayed enhancement had decreased, mostly in the basal-inferolateral segment (see Figure 1b and c). However, patchy midwall and subepicardial delayed enhancement was still present in 4 myocardial segments (i.e., the basal-inferolateral, mid-inferolateral, basal-inferior, and mid-inferior segments). Follow-up ECG did not show any abnormalities except for a minimally widened QRS complex compared to the pre-trial ECG and the persistence of incomplete right bundle branch block (SI File 4). In addition, ECG during a cardiac stress test did not show ST-T-segment changes, rhythm abnormalities, or other changes with respect to the pre-trial ECG. The cardiac stress test used a cycling protocol starting at 50 watt and with increasing steps of 20 watt per minute. He reached a maximally achieved power of 270 watt (i.e., 122% of expected for his age group and gender). He had an adequate increase in blood pressure and heart rate. His heart rate pressure product was 35,854 mmHg/min (normal is >25,000 mmHg/min). Metoprolol 25 mg once daily was stopped and the volunteer has remained without complaints in good condition.

Discussion

Clinically suspected acute myocarditis with typical MRI characteristics is reported in a healthy volunteer participating in a PfSPZ-CVac approach phase 1 clinical trial. The first myocarditis manifestations occurred 71 days after the last dose of PfSPZ Challenge, 44 days after the last dose of chloroquine, 57 and 31 days after receiving pre-travel vaccines, 12 days after CHMI by *P. falciparum*-infected mosquito bites, 3 days after the onset of a sore throat, and 1 day after diagnosis of *P. falciparum* malaria and initiation of treatment for malaria. The retrosternal chest pain [11], kinetics of increased troponin T plasma concentrations, ECG and echocardiogram findings, and MRI findings, which are consistent with the guidelines of the International Consensus Group on MR Diagnosis of Myocarditis [12], support the diagnosis of acute myocarditis. Moreover, improved myocardial function, disappearance of oedema and reduced delayed enhancement after almost 5 months correspond to the natural course of acute myocarditis; the residual delayed enhancement is consistent with contrast retention in fibrous tissue [13]. In addition, BNP and NT-proBNP were temporarily elevated and their elevation has also been found in patients with myocarditis and is associated with reduced left ventricular function [14,15].

The occurrence of the cardiac event relates in time with residual parasitaemia during curative Malarone treatment post-CHMI that might be suggestive of a causal relationship. A few cases of malaria and concomitant myocarditis have been reported in the literature, albeit restricted to patients with severe or fatal infection with *P. falciparum* [16-22] and *Plasmodium vivax* malaria [23]. In literature, myocarditis has never been reported in patients with uncomplicated *P. falciparum* malaria, even in those patients who present with *P. falciparum* parasite densities 20 to 30 times higher than the parasite density in this volunteer. In addition, troponin T plasma concentrations have never shown elevations

above background in an unselected group of 167 volunteers when daily measured after CHMI using a highly sensitive assay [personal communication by RW Sauerwein (Radboud University Medical Center, The Netherlands)]. Consistent with this finding, troponin T was very rarely (0.6%) elevated when assessed retrospectively in patients with uncomplicated *P. falciparum* malaria [24]. In contrast, 31–80.5% of African children with severe and/or fatal *P. falciparum* malaria exhibited high to very high levels of circulating cardiac proteins indicating myocardial injury and impaired left ventricular function [25].

Previously, a cardiac serious adverse event was reported in a female volunteer who participated in a phase 1 clinical trial in which she was immunized with a subunit, recombinant protein malaria vaccine (PfLSA3), underwent CHMI by mosquito bites, developed malaria, and was treated with Riamet® (artemether/lumefantrine) [26]. She was diagnosed with acute coronary syndrome 2 days after completing treatment, but myocarditis was considered a possible alternative diagnosis. Apart from 1 confirmed myocardial infarction in a male volunteer with an increased cardiovascular risk, who underwent CHMI but did not develop parasitaemia [3], there have been no other reports of cardiac complications in the approximately 2,000 subjects who have undergone CHMI since the 1986 report by Chulay et al. [1].

In the current case the cardiac event occurred during the 3 days when the subject was receiving curative Malarone treatment for *P. falciparum* malaria. There are no previous data indicating that antimalarial treatment with atovaquone/proguanil (Malarone®), or its metabolite cycloguanil causes myocarditis or any other significant cardiovascular toxicity [27]. Furthermore, the product monograph of Malarone does not mention cardiotoxicity or myocarditis, only palpitations and tachycardia.

The most common cause of acute myocarditis in a healthy young individual is a viral infection [28]. Numerous infectious pathogens can cause acute myocarditis [29,30]. Enteroviridae (including Coxsackie B) were responsible for 25–30% of cases in the past [31], but more recently, other viruses (including adenovirus, parvovirus B19, and hepatitis C) have also emerged as important cardiotropic pathogens [32]. In the current volunteer, diagnostic tests for the most common infectious causes of myocarditis were negative (Table 2 and 3). However, negative convalescent antibody titers do not exclude a post-infectious myocarditis. Furthermore, a throat swab, taken 2 days after the initial increase in troponin T contained rhinovirus, and rhinovirus has been occasionally associated with myocarditis [33,34]. Noteworthy, rhinovirus is often detected by PCR in asymptomatic subjects and a causal inference with symptomatic patients should, therefore, be made with caution [35].

The pathogenesis of myocarditis can be due to direct infection of the myocardium by a replicating pathogen, the host's specific immunologic response to such an infection [36], or a nonspecific immunologic response in a susceptible individual that could have been triggered in this case by the malaria infection. In most such cases one would expect to find markers of inflammation elevated. However, markers for nonspecific inflammation

and haemolysis, D-dimer, CRP, and LDH, were normal or only slightly elevated when the troponin T levels were highest (Table 1). Nonetheless, it is possible that an overall hyper-reactivity induced by the 6 standard vaccines (i.e., diphtheria, poliomyelitis, tetanus, parenteral typhoid fever, hepatitis A and hepatitis B) the volunteer received between the immunization period and CHMI, and the subsequent CHMI could have generated a hypersensitivity myocarditis. Such post-vaccination myocarditis has been rarely reported for the administered vaccines and usually manifests with fever and/or other nonspecific inflammatory symptoms within several days of the hyperimmunizations [37], which did not occur in this volunteer. Nonetheless, this explanation cannot be ruled out.

No systemic allergic reactions or local adverse events have occurred in the 20 volunteers, who have now received 3 intradermal (ID) injections of 7.5×10^4 PfSPZ of PfSPZ Challenge. Moreover, there have been no systemic allergic reactions among the 184 subjects who have received single doses of PfSPZ Challenge by the ID (n=84), intramuscular (IM) (n=70), and intravenous (IV) (n=30) routes in order to study the safety and infectivity of PfSPZ Challenge [8,9, and personal communication by SL Hoffman (Sanaria Inc., USA)], or among the 120 volunteers who have received up to 6 doses of 1.35×10^5 radiation-attenuated PfSPZ (PfSPZ Vaccine) ID (n=40), subcutaneously (SC) (n=40), or IV (n=40) [38,39]. Thus, it seems extremely unlikely that the parenterally administered PfSPZ or the phosphate buffered saline or human serum albumin with which the PfSPZ are administered contributed to the myocarditis or contain an immunologically sensitizing agent.

Myocarditis may also be triggered by toxins, alcohol, cocaine, chemotherapeutics, antibiotics, metabolic abnormalities, and other factors [29,30]. However, the volunteer denied excessive use of alcohol, and urine drug tests for cocaine, amphetamines and cannabinoids were negative, making such factors an unlikely explanation. Alcohol intake was not quantified during follow-up visits after CHMI, but volunteers were repetitively instructed to restrict alcohol intake. Since the urine drug test was performed 5 days after the first rise in troponin T, the detection of metabolites of cocaine and amphetamines after single use is quite limited at this time and could have been missed [40].

In conclusion, there are different possible causes for the myocarditis but a definitive cause in this case cannot be established. It is also possible that a combination of the above-discussed potential aetiological factors could have contributed to the development of this case of acute myocarditis.

Consent

Informed consent for publication of this case report was obtained from the volunteer who participated in this clinical trial.

Acknowledgements

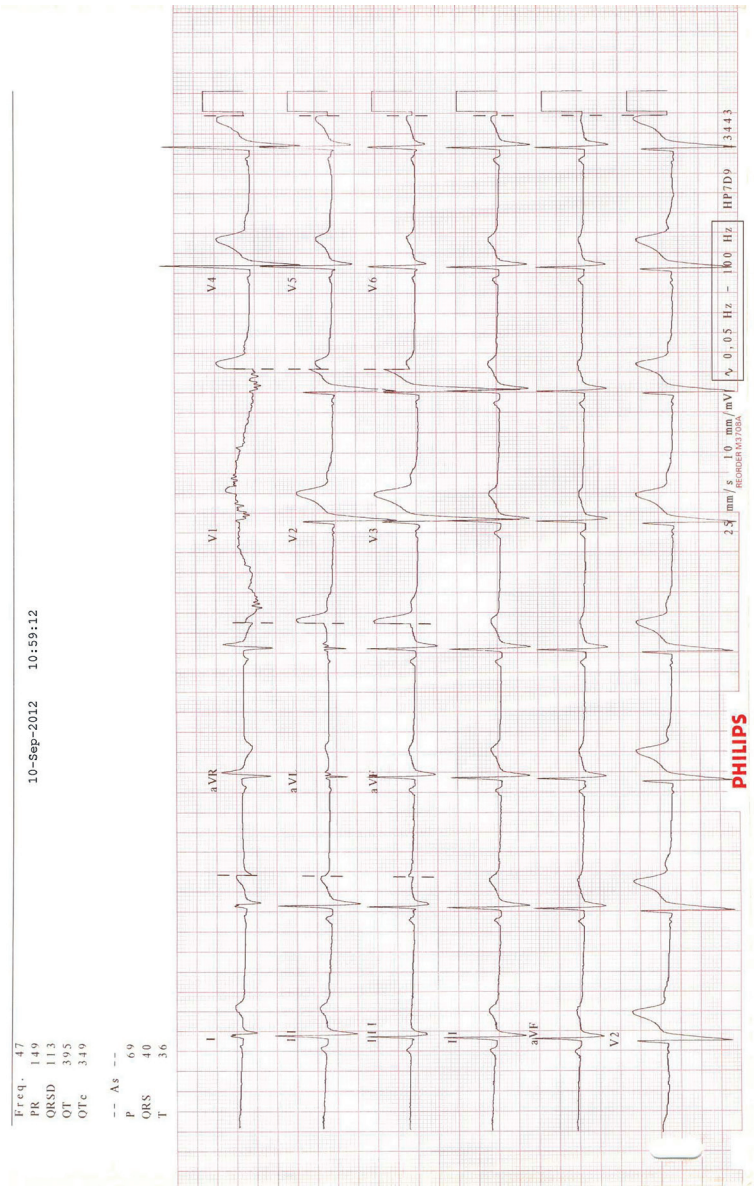
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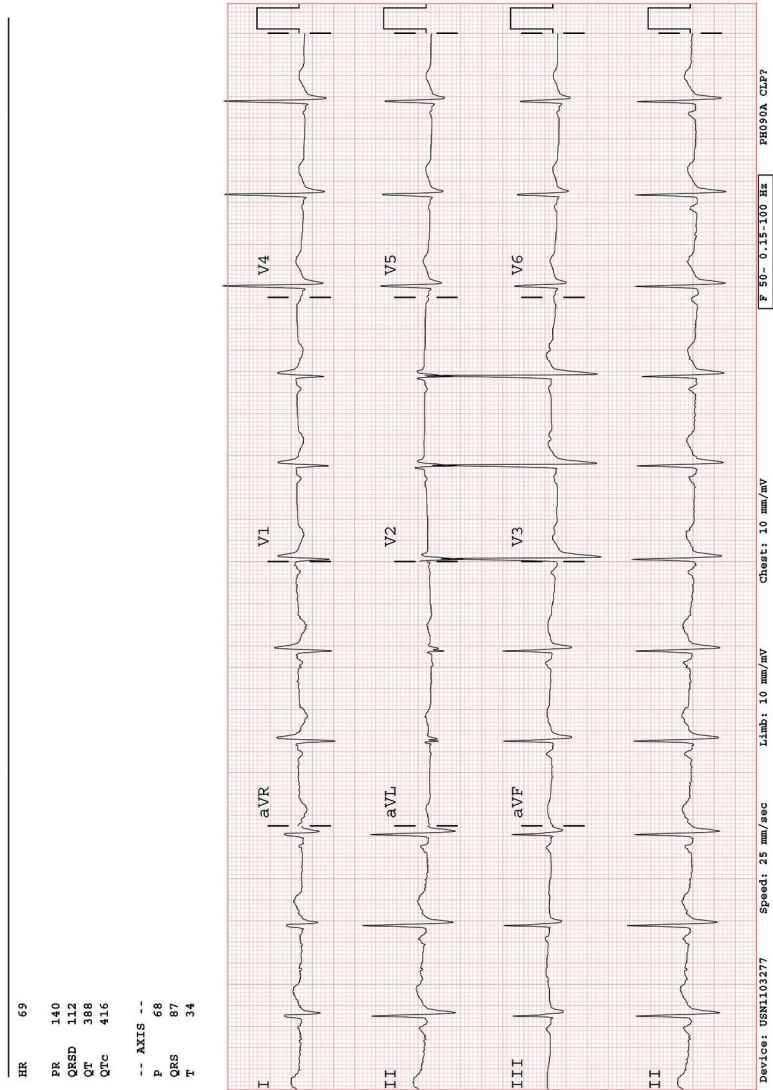
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Supporting Information



SI File 1 Electrocardiogram at screening visit before start of the clinical trial (10-SEP-2012, 10:59 AM) showing a normal variant of an incomplete right bundle branch block.

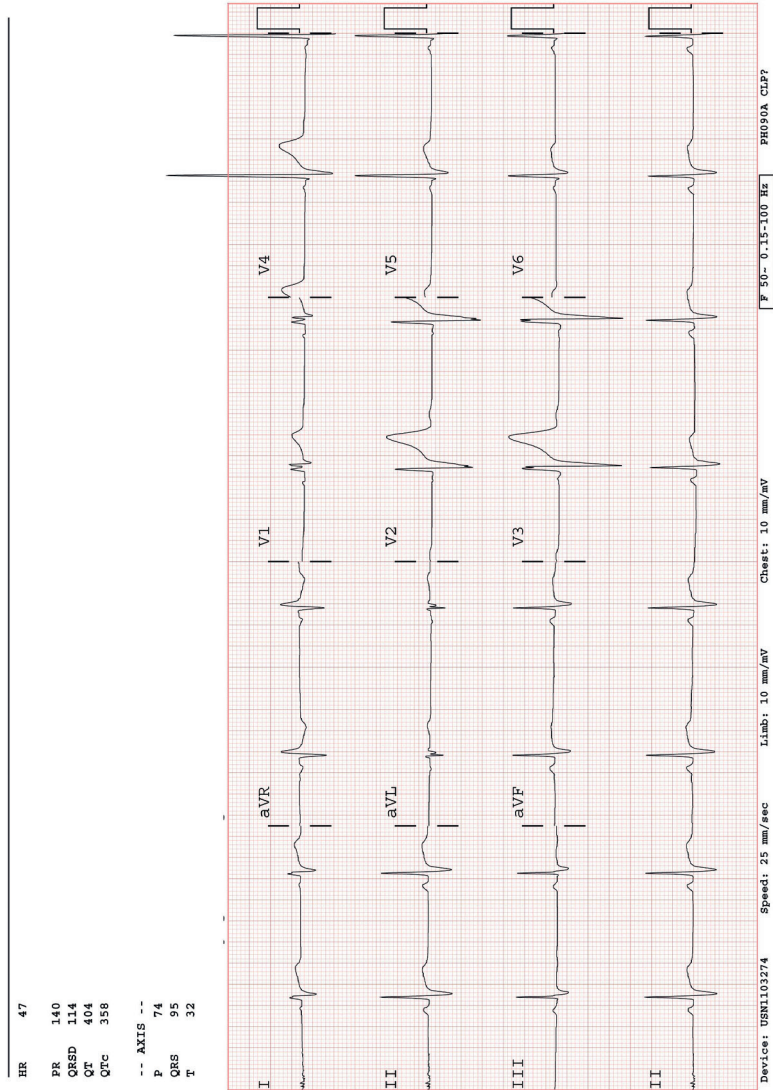
18-Feb-2013 19:41:40



SI File 2 Electrocardiogram on day 13 after CHMI (18-FEB-2013, 07:41 PM) showing mild repolarisation disturbances with diffuse ST-T-segment elevation.

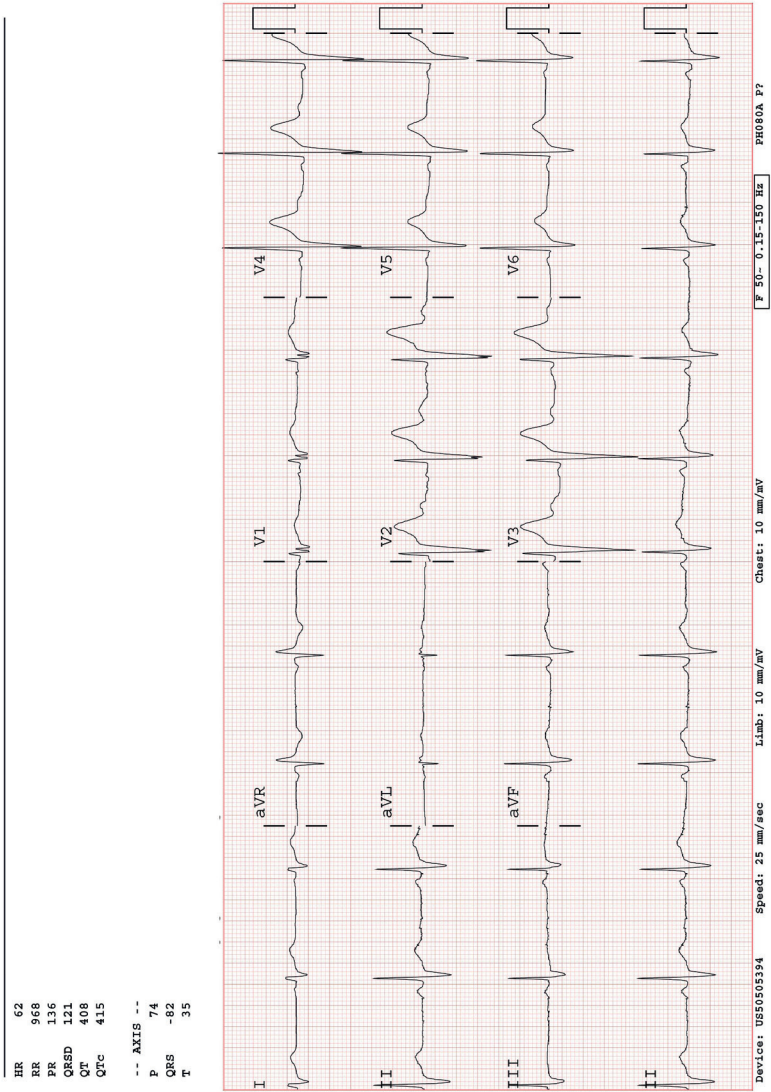


21-Feb-2013 12:13:36



SI File 3 Electrocardiogram on day 16 after CHMI (21-FEB-2013, 12:13 PM) showing normalization of the repolarisation disturbances compared to the previous ECG of 18-FEB-2013.

08-JUL-2013 09:48:09



SI File 4 Electrocardiogram on day 153 after CHMI (08-JUL-2013, 09:48 AM) showing no abnormalities except for the known incomplete right bundle branch block and a minimally widened QRS complex compared to the pre-trial ECG.



CHAPTER 9

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

The main objective of this thesis was to evaluate currently available and experimental treatment and vaccination strategies that have the potential to support malaria control, elimination, and eradication efforts now and in the future. The work described in this thesis focused on three main strategies:

1. Improving access and accuracy of diagnosis using malaria rapid diagnostic tests

Presumptive treatment of suspected malaria cases was a justified approach to combat malaria in a time when many regions were affected by high malaria risk and limited availability of diagnostic tools. When chloroquine and sulphadoxine-pyrimethamine were first-line antimalarial drugs, presumptive treatment of suspected malaria cases was also financially attractive because they were cheaper than the cost of malaria diagnosis. I examined the consequences of this approach now malaria prevalence and mortality are decreasing [1-6].

Countries in the east and south of sub-Saharan Africa have recorded patterns of decreasing malaria transmission [3,7,8] and malaria mortality rates dropped by 33% in the WHO African Region between 2000 and 2010 [9]. In general, and especially in the context of this changing malaria epidemiology, health workers may not always apply treatment according to test results in those sub-Saharan settings where microscopy is available [10-13]. Malaria rapid diagnostic tests (RDTs) are becoming increasingly available and may form an attractive alternative to microscopy to improve access to and accuracy of diagnosis and thereby support rational treatment of malaria.

In the study described in **Chapter 2**, we observed an immediate and evident effect of a policy change by the Tanzanian Ministry of Health and Social Welfare, comprising the provision of RDTs and advice to restrict antimalarial treatment to RDT-positive individuals. The significant reduction in antimalarial prescriptions after RDT implementation in two Designated District Hospitals (DDH) situated in low to very-low malaria endemic areas, was also observed in other studies particularly those where RDTs were introduced in presumptive treatment settings (**Chapter 3**). In general, the advantages of RDT implementation are harder to define when RDTs are introduced in settings that have used microscopy examination of blood smears (**Chapter 3**).

Our findings in **Chapter 2** are in line with the widely observed phenomenon that health workers often do not apply treatment according to test result [10-13]. This is often associated with the fear of missing low density infections that may later result in fulminant disease, although the limited available evidence indicates that withholding antimalarial therapy in febrile children with negative test results by microscopy, is likely to be safe [14]. In Sumve DDH and Rubya DDH (before RDT-implementation) clinicians frequently requested blood slides, but also frequently ignored negative slide results. In these hospitals up to 81% of the slide-negative individuals were treated with antimalarials. In Biharamulo DDH 79% of the children were treated with antimalarials without parasitological

confirmation, illustrating the persisting effects of long-held WHO-guidelines that promoted presumptive treatment of malaria in cases of fever [15].

At first instance it was encouraging to see an increase in antibiotic prescriptions after RDT-implementation, indicating consideration of alternative diagnoses and treatment options by medical staff. One may, however, question whether the observed rise in antibiotic use is proportionate or simply illustrates the replacement of irrational antimalarial use by irrational antibiotic use [16]. This phenomenon of (disproportionally) increased antibiotic use was seen in all studies where antimalarial prescription rates were reduced in malaria-negative patients [10,17-19] (**Chapter 3**), illustrating the considerable challenges to assist alternative diagnosis and treatment strategies for RDT-negative patients. Currently, there is little data on the spectrum of infections in patients presenting with symptoms of suspected malaria but a considerable part is probably self-limiting [20,21]. One study showed that the majority of febrile ($\geq 38^{\circ}\text{C}$) patients younger than 10 years of age attending outpatient clinics in Tanzania were most likely suffering from a viral illness [22], while another study showed that the majority (90%) of febrile RDT-negative children recovered safely without antibiotic treatment [23]. Thus, sending such patients home without antimalarial or antibiotic treatment in the absence of severe clinical signs could be a reasonable suggestion. It remains, however, a considerable challenge to distinguish between patients who are at risk for severe disease and therefore need antibiotic treatment and/or referral, and those where antibiotic treatment could be safely withheld. Development and supply of cheap and easy to use point of care tests for bacterial infections or biomarkers of severe infections, as are now increasingly available for malaria in the form of RDTs, will support medical personnel to rationally prescribe both antimalarials and antibiotics preventing spread of antimalarial as well as antimicrobial resistance.

In addition to the challenges of alternative diagnosis and treatment in RDT negative patients, the largest challenge for successful RDT implementation will be assuring and sustaining their supply at all levels of the health system. Particularly, areas where implementation of RDTs has shown a significant impact on antimalarial prescription rates are at risk of situations where health staff will regress to their former diagnostic and drug-prescribing behaviour when RDTs are irregularly supplied. With the increasing number of low endemic settings following successful malaria control, adequate and sustained coverage of RDTs is mandatory for rational antimalarial treatment.

In Rubya DDH, situated in a remote, rural, very-low malaria endemic area, we observed that RDTs provided by the Tanzanian government were out of stock shortly after initial roll-out (**Chapter 2**). Unfortunately, this is not uncommon. Particularly in rural areas, where access to services is often low but demand for services is highest [9], drug stock-outs are common [12,24-26] and supply is one of the biggest challenges facing the health system. Only few studies have examined supply chain characteristics and factors that affect stock levels and availability of essential health commodities at health centres. Main drivers of RDT stock-outs are most probably suboptimal supply, inaccurate inventory of existing

stock in stock cards, and poor system design [27]. RDT stock-outs will not only affect health centres, but also practice of community health workers (CHWs) who increasingly use RDTs and rely on nearby health facilities for their RDT stock supply [28].

A constant supply of RDTs is a massive and costly logistical undertaking and although RDTs are more robust than ever before, they are perishable items which need to be delivered under strict storage and transport conditions to allow efficient use. Furthermore, it is important that the private for-profit sector is included into this undertaking. This sector plays a key role in delivering services across most of Africa and the majority of suspected malaria episodes are initially treated by private health workers [29,30]. Data from a limited number of countries suggest that neither microscopy nor RDTs have penetrated the private healthcare sector [9,31], hampering the implementation of the new parasite-based policy.

Simply making RDTs available in all health care sectors will not directly lead to high uptake of the tests or adherence to results by health workers [32]. Health workers are often insufficiently trained and may distrust negative test results, have little confidence or resources to treat alternative causes of fever, and are influenced by the perception of patients demanding for antimalarial drugs (**Chapter 3**). These challenges seem to persist even when highly sensitive and specific RDTs are used [16] and when evidence suggests that adhering to RDT results does not have a negative effect on health outcomes [32]. Training and regular retraining using proven supportive interventions which take staff turnover into account, alongside education of community members is urgently needed.

However, there is little evidence on how to optimally design health worker training to improve adherence to current malaria treatment guidelines including use of RDTs. Only few studies have assessed interventions intended to change health workers practice when introducing RDTs. Recent evidence from a cluster randomized trial in Cameroon shows that a mindset-oriented, interactive training approach results in a significant reduction in antimalarial overtreatment and increases the percentage of febrile patients being tested for malaria, compared to the “standard” conventional, knowledge-based and skills-oriented, didactic training approach. The new training approach covered three additional modules targeting improvements in quality of care. These modules focussed on how to adapt to a change in policy, on discussions about professionalism, and on improving communication skills to patients including managing patients’ expectations [33]. Novel interactive training methods like the one described in this study are the way forward to bring about the behaviour change needed to support the rollout of RDTs.

If positive trends continue and malaria transmission will continue to decrease in many sub-Saharan areas, the number of (very) low transmission and elimination settings will increase. These settings present new challenges that will demand new diagnostic tools more sensitive than current RDTs and new strategies, i.e. a change from passive case detection to “active” case detection to detecting the highest possible fraction of infections in the general population.

In low endemic settings the proportion of malaria-infected individuals who harbour parasites at densities below the microscopic threshold for detection is considerable [34] and the currently available RDTs do not detect all malaria-infected individuals. Research aimed towards increasing the sensitivity of existing RDTs may not change this because of the limitations of the currently available technology. In low endemic and elimination settings, the diagnostic sensitivity (the proportion of target cases detected by the test) for passive as well as active case detection should be $\geq 99\%$, and the analytic sensitivity (the detection threshold against the marker of the infective agent in controlled conditions) should be < 1 parasites/ μL , particularly for active case detection [34,35].

One operationally attractive approach to use RDTs, with shortcomings in sensitivity to detect asymptomatic infections, would be to use RDTs to identify geographical clusters of malaria infections. RDT-positive cases are likely to be surrounded by (sub-patent) infections [36] and RDT-positive individuals can thus form sentinel cases for mass screening and treatment using new diagnostics that can detect very low levels of *Plasmodium* in the blood (1 parasite/ μL) of asymptomatic individuals who may contribute to continuing malaria transmission [37,38].

With the current focus on malaria elimination and the increasing awareness of the contribution of low density infections to onward malaria transmission, the need for more sensitive field-ready diagnostic tools for mass screening and surveillance to monitor transmission reduction and ensure elimination, is increasing. Detecting circulating parasites by demonstrating parasite DNA through amplification of ribosomal RNA genes by PCR assays is currently the most sensitive diagnostic method. However, PCR capacity is limited in resource-poor settings due to the need to avoid contamination which leads to false-positive results, requiring a very high standard of laboratory practice [39,40]. The use of centralized PCR-specialized labs where field samples obtained by active or passive case detection are sent and analyzed, should be evaluated, with a particular focus on logistics (sample transfer, time to test result and feedback to field team, communication between centralized lab and field team).

Another molecular detection method based on DNA amplification is loop-attenuated isothermal amplification (LAMP). This promising method, which amplifies DNA (usually mitochondrial) with a single thermal cycle, has the potential to reduce the training and infrastructure requirements of molecular diagnosis [41-43]. LAMP would allow the timely feedback of results needed for case management, but could also be used for mass screening and surveillance detecting very low parasite densities. However, LAMP has not yet been adequately field tested for wide-scale use or developed in a format suitable for the processes of high sample numbers, and sensitivity could be further optimised. In a remote Ugandan clinic LAMP achieved sensitivity similar to that of single-well nested PCR, but showed false-negative results at parasitaemia levels detectable by 3-well nested PCR [44].

In conclusion, expanding access to RDTs on the scale needed to achieve universal coverage requires retraining of public, private, and retail sector providers as well as sustained

supplies and quality assurance. In the increasing number of (very) low transmission and elimination settings RDTs will not (and may probably never) be able to detect all (asymptomatic) malaria infections. Therefore, new diagnostic tools capable of identifying very low parasite densities should be developed for case management, mass screening and surveillance, and use of currently available highly-sensitive tools like PCR should be evaluated in resource-poor settings. Furthermore, as malaria case incidence will drop, research is required into diagnostics for non-malarial febrile diseases and into the integration of these tests into health systems.

2. Mosquitocidal additions to current antimalarial treatment to prevent transmission

Gains in malaria control over the last decade are severely threatened by widespread insecticide resistance in *Anopheles* vectors [45-47] and emerging artemisinin-resistant *P. falciparum* [48,49], posing a major challenge to worldwide control and elimination. New vector-targeted interventions that reduce malaria transmission are needed, in particular a broader range of insecticides with novel modes of action and control methods that target vector species regardless of their biting habits, i.e. act against outdoor feeding and resting vectors [50]. Moreover, the high vectorial capacity [51] of malaria vectors in sub-Saharan Africa should be reduced [50,52]: decreasing *Anopheles* daily survival rates will show the greatest effect on malaria transmission [53,54]. Our findings in **Chapters 4 and 5** address these issues and show great potential of ivermectin (IVM) as a powerful new addition to the toolbox of antimalarial measures.

Results of animal studies (**Chapter 4**) show a pronounced effect of IVM on mosquito mortality: 70-100% of mosquitoes died within 72 hours after taking a blood meal within 2 days after IVM treatment. This strong, short-lived mosquitocidal effect supports several previous observations of IVM [55-57], with more detailed daily assessments and a larger number of mosquito observations. The short period of killing activity suggests that IVM may be less attractive as a stand-alone drug for mass drug administration (MDA) campaigns that aim to reduce malaria transmission. IVM may, however, be a potent addition to antimalarial drugs in MDA campaigns or to prevent transmission shortly after treatment of symptomatic malaria cases by reducing the likelihood that mosquitoes that feed on gametocytaemic human hosts live long enough to become infectious to other humans. With this rationale, we designed a clinical trial, described in **Chapter 5**, in which IVM was added as transmission-blocking drug to artemisinin-combination therapy (ACT). Findings confirmed our hypothesis: the survival of two major malaria vectors in sub-Saharan Africa, *An. gambiae* and *An. funestus* was significantly reduced in the study groups receiving AL-IVM. The mosquitocidal effect of IVM was apparent three to seven days after a single dose depending on volunteer gender with a more pronounced effect when mosquitoes fed on blood from female participants.

This trial was the first ever to formally confirm the safety profile of IVM in combination with ACT - in this case AL - in a small group of malaria-infected individuals. The AL-IVM combination was safe without evidence that co-administration of IVM affected the bio-availability of lumefantrine. IVM already had an established safety profile which allowed its use in MDA campaigns for onchocerciasis and treatment of lymphatic filariasis in Africa [58]. The mode of action of IVM as mosquitocidal drug depends on IVM activating glutamate-gated chloride channels in neuronal and neuromuscular tissues of invertebrates, thereby causing flaccid muscle paralysis that can lead to death of the insect [59-61]. Glutamate-gated chloride channels do not exist in humans and orthologous mammalian channels are distantly related and found only in the human central nervous system, where the blood-brain barrier limits drug access [62,63]. No significant association has been found between IVM plasma levels and adverse events [64], and doses up to 10 times the approved limit are well tolerated by healthy volunteers [65], providing little concern for drug toxicity even at high doses. This may be beneficial for future use, since our findings indicate that higher, repeated doses or sustained presence of IVM may be needed for maximal mosquitocidal effect. Modelling results indicate that increasing the duration of the mosquitocidal effect of IVM will have a greater impact than increasing the magnitude of the mosquitocidal effect on reducing infectious vector density and host parasite prevalence [66].

Adverse events related to IVM have been usually associated with intensity of microfilarial infection and primarily characterized as classical (mild) Mazzotti reactions to degenerating microfilariae [67]. However, in persons with high levels of *Loa loa* microfilariæ (>30,000 microfilaria/mL) IVM can induce excessive forms of the normally expected Mazzotti reactions, i.e. serious (neurological) adverse events including encephalopathy and coma [67-69]. This is a drawback if ACT-IVM were to be implemented in countries where loiasis is or may be endemic (Angola, Cameroon, Central African Republic, Congo, DR Congo, Equatorial Guinea, Gabon, and potentially parts of Chad, Ethiopia, Nigeria and Sudan). On the other hand, prevalence of this parasitic disease is still very low even in these countries [70] and prior screening using efficient and sensitive methods such as the RAPLOA assessment tool developed by the World Health Organization [71] and parasitological assessment by microscopic examination of a blood film [69] could be feasible.

The activation of glutamate-gated chloride channels does not only provide an excellent safety profile but also represents a novel model of action [59] circumventing the issue of emerging resistance to other currently used insecticides. Furthermore, as a systemic drug, IVM is ingested by all biting mosquitoes and so it will equally target indoor and outdoor-biting mosquitoes, as well as those with crepuscular activity, thereby avoiding the selective survival of outdoor biters that has been observed after the wide-scale deployment of bed nets [72]. Finally, the activity of IVM targets the most influential variable of vectorial capacity, the probability of mosquito survival through one day [53,54].

Use of single IVM-only MDAs is not expected to have a long-term effect on malaria parasite transmission [56,73]. Field data only show significantly increased mortality of wild *A. gambiae* for up to six days [56] and large reductions in sporozoite rates for at least 2 weeks after IVM MDA [73]. Modelling results suggest that IVM MDAs might only be able to significantly reduce malaria parasite transmission in a community if given once a month or more frequently, and probably only in areas with seasonal malaria transmission, such as the Sahel, or during epidemics [63].

We envisage a complementary role of IVM in malaria control, elimination and eradication efforts by combining it with antimalarials such as ACT, which rapidly clears asexual parasites and developing gametocytes but leaves mature *P. falciparum* gametocytes largely unaffected; a proportion of *P. falciparum* parasite carriers may transmit malaria after successful ACT treatment [74,75]. IVM could be an extremely valuable and safe alternative for or in addition to gametocytocidal drugs to reduce malaria transmission post-ACT to prevent the spread of artemisinin-resistant parasites throughout the world [76]. The most powerful approach to implement ACT-IVM in community campaigns to reduce malaria transmission depends on the previously mentioned challenges in identifying all malaria-infected individuals who contribute to malaria transmission. With the large proportion of submicroscopic infections in all settings, especially low endemic settings [34], restricting ACT-IVM to RDT positive individuals may have negligible sustained effects on malaria transmission [66]. There are three options to use ACT-IVM to bring down community transmission intensity: i) ACT-IVM may be distributed to parasite positive individuals in a mass screening and treatment (MSAT) approach where molecular diagnostics are used to screen individuals regardless of symptoms; ii) RDTs are used to identify high density parasite positive individuals who are indicative of clusters of asymptomatic (often submicroscopic) infections that can then be identified by more costly and laborious molecular diagnostics and treated with ACT-IVM; iii) a MDA approach where ACT-IVM is distributed to the entire community regardless of malaria diagnosis or symptoms. Although ACT-IVM MDA will result in unacceptable costs and drug-pressure on parasite populations if repeatedly performed, it may play a role in selected settings where elimination has nearly been achieved or where drug resistance needs containment [66,77]. Modelling results suggest that including mass IVM administration alongside mass screening and treatment (MSAT) or MDA with an ACT can substantially increase reductions in *P. falciparum* malaria transmission. This transmission effect is obtained through the effect IVM has on vector mortality [66].

Before IVM can be recommended for malaria control, further studies must be conducted to provide definitive evidence of its role in any type of malaria control strategy (Table 1). The ultimate evidence will come from cluster-randomized community trials with single or repeated IVM MDA preferably with addition of antimalarial MSAT or MDA. More modest studies to provide further evidence on the potential role(s) of IVM should directly compare membrane feeding with skin feeding, since cumulative mosquito mortality may

be higher after direct skin feeding [55]. There is evidence that IVM concentrations in different tissues may vary [78], but it is currently unknown if the observed difference in mosquito mortality between membrane and skin feeding is due to different IVM concentrations in capillary versus venous blood. Furthermore, it was recently suggested that sublethal IVM concentrations may affect sporogony in colonized *An. gambiae* s.s. mosquitoes infected with cultured *P. falciparum* parasites [79]. We did not observe a sporonticidal effect in our study, neither *in vivo* or *in vitro*. Since the attractiveness to use IVM will be also dependent on a possible sporonticidal effect that may reduce malaria transmission for a longer period than predicted based on anti-mosquito effects alone, it will be necessary to clarify the reasons for these conflicting results and potentially validate the results of the study by Kobylinski and colleagues with natural *P. falciparum* isolates and wild-caught anophelines.

Table 1 Challenges and future research for implementation of IVM

Large-scale community trials	<ul style="list-style-type: none">- Single or repeated IVM MDA + antimalarial MSAT or MDA- Effect of IVM MDA in diverse habitats containing different vectors and malaria ecologies
IVM dosage determination and spacing	<ul style="list-style-type: none">- Comparing membrane feeding with (human) skin feeding- Simultaneous mosquito feeding and measurement of IVM concentration in human plasma- Development of a safe, single-encounter, long-lasting IVM formulation
IVM resistance	<ul style="list-style-type: none">- Establishment of resistance-surveillance in communities where IVM will be introduced for malaria control- Explore combination of IVM with a second antihelmintic

IVM, ivermectin; MDA, mass drug administration; MSAT, mass screening and treatment.

Successful implementation of any type of IVM MDA would require good understanding of the transmission intensity and seasonality pattern of the particular target area to ensure that treatment rounds are conducted at optimal times and that interruption of transmission is feasible. Effects of (any type of) IVM MDA will have to be compared in diverse habitats containing different vectors and malaria ecologies. Since IVM is of utmost importance for control of onchocerciasis, lymphatic filariasis and treatment of some soil-transmitted helminthes [80], increased exposure of these parasites to IVM might lead to resistance and threaten the success of control programmes. Therefore, surveillance strategies must be established in areas where IVM is introduced as a malaria control measure. Combining IVM with a second antihelmintic drug might be effective to delay resistance development in soil-transmitted helminthes.

3. Protection against malaria by inoculation of whole sporozoites

In 1967, Nussenzweig et al. reported the first successful proof-of-concept studies of a live attenuated, metabolically active sporozoite vaccine in a rodent malaria model [81]. This landmark finding formed the basis for whole sporozoite immunization, which proved to provide protection against malaria challenge infection in humans in the 1970s [82-89]. Results by Hoffman et al. expanded these findings, showing protection against challenge infection in 24 of 26 volunteers immunized by irradiated mosquitoes carrying *P. falciparum* sporozoites [90]. This sterile protection most likely was based on pre-erythrocytic immunity acquired by irradiated sporozoites that arrest early after liver cell invasion providing liver-stage antigen exposure [90]. However, the requirement of a minimum of 1,000 bites by irradiated mosquitoes during five or more immunization sessions in order to successfully induce sterile immunity in humans precluded this method from routine immunization and the pursuit of a whole sporozoite malaria vaccine was largely abandoned.

In the past years, there has been renewed interest in the whole sporozoite immunization as a result of the cryopreservation of sporozoites by Sanaria Inc. [91] and the highly effective CPS (ChemoProphylaxis and Sporozoites) immunization model [92,93]. Using the CPS-approach, healthy malaria-naïve volunteers can be fully protected against a mosquito challenge infection with a homologous *P. falciparum* strain for up to more than two years after three immunizations under chloroquine prophylaxis by bites from 12–15 *P. falciparum* sporozoite-infected mosquitoes at monthly intervals [92,93]. The unprecedented efficacy of the CPS immunization model is represented by the low dose sufficient to induce protection compared with the irradiated sporozoite approach [90]. In the latter approach, asexual blood stages never occur and the apparent lack of blood-stage immunity was firstly shown in the 1970s in a single irradiated sporozoite-immunized volunteer challenged with blood-stage parasites [85].

In CPS immunization, transient low-level blood-stage parasitaemia does occur [92], because chloroquine only kills developing blood stages of *P. falciparum* without affecting sporozoites or liver stages [94]. Consequently, the host's immune system is exposed to a relatively broad repertoire of antigens, including sporozoite, liver-stage, and blood-stage antigens [92,95] of which many are shared between stages [96]. As well as posing the question whether this great array of antigens is the main reason for the potency of CPS-immunization, the exact mode of protective action remains uncertain: protection may be mediated by immune responses against liver stage parasites, blood stage parasites or a combination thereof [97].

In **Chapter 6** we have shown that CPS immunization does not protect against an intravenously administered challenge with *P. falciparum*-infected erythrocytes. This complete absence of apparent functional blood-stage immunity emphasizes the need for a 100% effective liver-stage immunization if this model is to be translated into an actual

vaccine. Especially in people without natural immunity, a single surviving parasite caught in an endemic area may be sufficient for the infection to proceed to the blood stage causing clinical disease. A selective group of non-immune subjects, such as military and travellers, is expected to be the primary target group for a CPS-based vaccine in the near future. Thus, even when a CPS-based vaccine would provide a high rate of sterile protection, the absence of functional blood-stage immunity may cause safety concerns and warrant a continued need for antimalarial prophylaxis. Another issue to deal with is co-administration of the antimalarial drug. Currently this is chloroquine, although mefloquine was recently shown to be equally effective in the CPS immunization model (E. Bijker et al., submitted). It is crucial that full attenuation is independent from compliance and pharmacokinetics of the blood-stage killing drug used and that the drug formulation should have a sufficient half-life. Finally, extensive post-vaccination follow-up might be necessary due to the inability to carry out viability checks before the vaccine is administered. However, progress in the development of in vitro hepatocyte assays to reliably and repeatedly check the potency of sporozoites should solve this issue.

The biggest impediment for direct practical application of CPS-immunization is its dependence on inoculation of sporozoites by mosquito bites. The first study ever to address this issue is described in **Chapter 7** where we replaced mosquito bites by intradermal needle and syringe injections containing sporozoites, an approach called *PfSPZ-CVac* (*PfSPZ-Chemoprophylaxis Vaccine*). We used the *PfSPZ Challenge* product of the biotechnology company Sanaria Inc. (USA) which consists of infectious, aseptic, purified, vialled, cryopreserved *Pf* sporozoites manufactured according to Good Manufacturing Practice [98,99].

Results of this first phase I trial showed that the current intradermal immunization regimen of *PfSPZ-CVac* immunization was safe and well tolerated. Furthermore, a cardiac SAE occurred from which the definitive aetiology could not be established (**Chapter 8**). The current immunization regimen did not confer protection against a homologous challenge infection in healthy malaria-naïve volunteers. In contrast to previous CPS studies [92](**Chapter 6**), parasitaemia was never observed after an immunization in any of the *PfSPZ-CVac* recipients, plausibly reflecting insufficient liver stage maturation and presumably sub-optimal parasite exposure. Under these conditions the host's immune system is underexposed to the broad array of antigens necessary for induction of protective pre-erythrocytic immune responses [100,101]. Differences in efficiency of administration (route/method) and infectivity/fitness of sporozoites between CPS and *PfSPZ-CVac* immunization are likely responsible for the observed absence of blood-stage parasitaemia as a reflection of unsuccessful liver-stage maturation.

With the application of multiple intradermal injections of sporozoites by needle and syringe we tried to approach the way anopheline mosquitoes successfully deliver sporozoites: before a capillary is found the female mosquito probes the dermal skin several times, while injecting tiny bits of sporozoite-containing saliva [102]. Evidence supporting multiple

intradermal injections was derived from murine data demonstrating that both intramuscular and intradermal routes increased liver loads when using smaller volumes and injections at multiple sites [103]. However, despite the apparent similarity to mosquito bite delivery it is evident that the current method of 6 intradermal sporozoite injections is still clearly different from natural conditions: i) mosquitoes deliver a proportion of sporozoites intracapillary and a proportion intradermally [104]; ii) sporozoites are embedded in mosquito saliva when inoculated, a component that may improve infectivity [105]; and iii) the volume of injections by mosquito is considerably lower than will ever be reached by needle and syringe. Injecting smaller volumes of e.g. 1 μ L in future trials may prevent sporozoites from 'swimming' in pools of intradermally injected fluid, providing them with the possibility to adhere to dermal cells and subsequently migrate to capillaries and lymphatic vessels [106]. Furthermore, increasing the number of injections may improve chances that sporozoites find a dermal capillary. For this a novel injecting device should be designed that simultaneously or consecutively injects very small volumes of sporozoites. Apart from optimizing route and method of immunization, increasing the infectivity of cryopreserved sporozoites by Sanaria Inc. and consequently optimizing effective dose will be key for clinical development of PfSPZ-CVac.

It will take more time to translate CPS-immunization into an effective and implementable malaria vaccine. Apart from impediments mentioned before, the genetic variation of parasite clones in the field may form an obstacle [107]. There is some evidence from the 1970s and 1990s that whole sporozoite immunization induces protection against heterologous *P. falciparum* challenge: heterologous *P. falciparum* infection by mosquito bite, after immunization with radiation attenuated *P. falciparum* sporozoites by the bites of >1,000 mosquitoes, was performed 7 times in 4 volunteers with protection in all 4 volunteers [90]. Furthermore, we have recently obtained supportive evidence for existence of heterologous protection after CPS immunization. Two out of 12 subjects, who had previously received CPS immunization with 3x5, 3x10 or 3x15 infected bites and were homologously challenged, were protected against heterologous re-challenge with significant delays in pre-patent period in most of the volunteers previously protected against the homologous challenge (R. Schats et al., submitted). To confirm these promising data and understand the reasons for cross-protection and failure to achieve cross-protection, a new clinical CPS-trial primarily aimed at assessing heterologous protection has been designed.

Apart from CPS-immunization, sporozoites attenuated by targeted gene disruption, of which the favourite candidates are genetically attenuated late liver-stage-arresting parasites, are being evaluated as whole-parasite vaccines [108]. In mice, this type of vaccine induced high levels of cross-species protection and complete protection when administered by intradermal or subcutaneous routes [109]. Such results still need to be generated in humans.

Another way of using the CPS-immunization model in the future, would be to translate it into a natural immunization strategy in malaria endemic areas. Here, chloroquine

administration would not only have a direct prophylactic effect, but could also provide protective efficacy to malaria after cessation of the drug. Ideal areas to test the feasibility and efficacy of this strategy would be those with a short but intense malaria transmission season with similar inoculation rates as challenge infections [110]. Particularly in these areas, where the exposure to pre-erythrocytic antigens will be high while blood-stage infections are controlled by chloroquine, protective efficacy could be assessed in the next peak transmission season by measuring incidence of clinical malaria, prevalence of malaria parasitaemia and parasite densities.

A major roadblock for the applicability of natural immunization is the prevalence of parasite populations with resistance to chloroquine [111], necessitating alternatives to chloroquine monotherapy. Murine immunization studies with *P. berghei* in combination with pyrimethamine or primaquine prophylaxis show promising results [108]. Furthermore, use of an ACT like dihydroartemisinin-piperaquine is potentially interesting and could be assessed in the lab and in the field. Dihydroartemisinin kills *Plasmodium* blood-stages by targeting the food vacuole in which the parasite concentrates haem [112]; the mechanism of action of piperaquine is currently unknown, but it is believed to resemble that of chloroquine.

Although the natural immunization approach might be more feasible for the near future, a CPS-based (or other type of) vaccine would probably hold larger promise to support malaria elimination initiatives. In (very) high transmission areas sporozoite inoculation rates may rapidly reach the threshold for a natural immunization strategy. In moderate, low and elimination areas, natural immunization could also be applied but should be accompanied by regular immunizations with sporozoites to enhance rapid development of herd immunity.

Conclusion

Each of the treatment and vaccination strategies described in this thesis has the potential to make a significant contribution to malaria elimination (and eventually eradication). To achieve malaria elimination, i) access to highly sensitive and accurate diagnosis is essential in order to find all malaria-infected individuals, and diagnostics should be suitable for mass screening, ii) parasite positive individuals should be treated and onwards transmission should be prevented, and iii) gains should be sustained and herd immunity should be induced in low endemic and elimination areas. Strategies described in this thesis, could be applied into this three-step approach: i) RDTs could increasingly be used to identify geographical clusters of malaria infections ii) that could then be targeted with IVM-ACT MSAT or MDA to prevent the spread of (resistant) parasites to surrounding populations, and eventually iii) a whole-sporozoite vaccine (once developed) could protect the population against future malaria outbreaks.

Interrupting malaria transmission worldwide is one of the greatest challenges for international health and development communities. Even though substantial progress

has been made in reducing malaria prevalence, further scaling up of control with currently available knowledge, tools and strategies will most likely fall short of eliminating malaria in most endemic settings [113]. The efficient transmission of malaria, with a single malaria infection giving rise to dozens or even hundreds of new malaria cases [114], makes it tremendously challenging to achieve and sustain local malaria elimination. To eliminate malaria from all regions of the world, i.e. malaria eradication, additional challenges exist with a major role for operational limitations (e.g. underperforming health services/systems, insufficient human resources, inadequate tools to interrupt transmission in high transmission areas, political unrest) and heterogeneity in malaria transmission between areas (e.g. different vector, parasite, social and environmental factors) indicating that there is no single strategy that works everywhere. Furthermore, the parasite and the vector are always evolving, indicating that there are - and always will be - significant threats to the progress made. Examples are the emergence of artemisinin resistance, the consequent need for more vigorous containment measures [76], and the increased mosquito resistance to insecticides warranting the need for new vector control strategies [46].

Continued financial and political support to prevent resurgence of malaria in countries that have brought the malaria burden down as well as development of new and improved tools and strategies, and their implementation in various settings is crucial to achieve eradication, hence the impetus to explore transmission-blocking and live attenuated parasite vaccines as well as anti-vector measures targeting novel processes. The *Plasmodium* parasite and the *Anopheles* vector offer many targets for intervention that are currently still unknown. A better insight into the genome sequences of both will provide a better understanding of host-parasite interactions and enable us to design new interventions. The future of malaria control and eradication efforts will be highly dependent on how well the scientific and public health communities, policy makers and local governments can work together to extend the effective life span of our existing tools while discerning new interventions that interrupt the complex life cycle of *Plasmodium* parasites.

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CHAPTER 10

SUMMARY

Interrupting malaria transmission worldwide is one of the greatest challenges for international health and development communities. Even though substantial progress has been made in reducing malaria prevalence, further scaling up of control with currently available knowledge, tools and strategies will most likely fall short of eliminating malaria in most endemic settings. This is partly due to constant threats to current progress, such as emergence of antimalarial drug resistance and vector mosquito resistance to insecticides. Consequently, need for more potent tools to contain dissemination of resistant parasites and accelerated development of vaccines that can impact on malaria incidence, disease, and death remains a high priority.

In this thesis, I evaluate currently available and experimental treatment and vaccination strategies that have the potential to support malaria control, elimination, and eradication efforts now and in the future. The work described in this thesis can be grouped into three main sections.

- A. Improving access and accuracy of diagnosis using malaria rapid diagnostic tests, a current strategy
- B. Mosquitocidal additions to current antimalarial treatment to prevent transmission, a near future strategy
- C. Protection against malaria by inoculation of whole sporozoites, a future strategy

In **Section A** we show that malaria rapid diagnostic tests (RDTs) can support rational treatment of malaria, as they provide parasite based diagnosis in presumptive treatment settings and are an attractive easy-to-use alternative to microscopy. In **Chapter 2** we observed an immediate and evident effect of a policy change by the Tanzanian government, comprising the provision of RDTs and advice to restrict antimalarial treatment to RDT-positive individuals. There was a significant reduction in antimalarial prescriptions after RDT implementation in two district hospitals situated in low to very-low malaria endemic areas in Tanzania. In **Chapter 3** we show that this reduction was also observed in other studies, particularly those where RDTs were introduced in presumptive treatment settings, but that the advantages of RDT implementation are harder to define when RDTs are introduced in settings that have used microscopy examination of blood smears. Furthermore, we highlight the World Health Organization's (WHO) initiative *T3: Test. Treat. Track*, which centres on the use of RDTs to address the widespread problem of poor access to diagnostic testing and antimalarial treatment. We argue that the biggest challenge in RDT implementation will be to provide adequate and sustained supplies of RDTs and appropriate training to all health workers in endemic areas.

In the **Section B** we present an alternative approach to prevent post-treatment malaria transmission. The transmission of malaria parasites from humans to mosquitoes (and subsequently to other humans) depends on the presence of mature sexual parasite forms

(gametocytes) in the peripheral blood. Gametocytes are relatively insensitive to current first line antimalarial drugs that therefore need to be supported by specific transmission-blocking drugs. The search for malaria transmission-blocking drugs has so far focused on drugs that clear gametocytes. We discovered that ivermectin (IVM, a drug widely used against onchocerciasis) can prevent post-treatment malaria transmission in an unconventional way: by killing the vector, the *Anopheles* mosquito, instead of killing the gametocyte. In animal studies described in **Chapter 4** we discovered a pronounced but short-lived mosquitocidal effect of IVM. In **Chapter 5** we confirm these findings in humans by performing a double-blind, placebo-controlled trial in Burkina Faso, in which we show that the combination of IVM with the current first line antimalarial drugs is safe and significantly reduces the survival of malaria-transmitting *Anopheles* mosquitoes. The mosquitocidal effect of one and two doses of IVM in combination with first line antimalarial drugs resulted in 27% and 35% reductions, respectively, in estimated malaria transmission potential during the first week after initiation of treatment. We argue that IVM can be recommended for malaria control in the near future, but that further studies must be conducted to provide definitive evidence of its role in any type of malaria control strategy. The ultimate evidence will come from cluster-randomized community trials with single or repeated IVM mass drug administration preferably with addition of mass drug administration of first line antimalarial drugs.

In the **Section C** we describe two controlled human malaria infection studies in which we immunized malaria naive volunteers under chloroquine chemoprophylaxis with *Plasmodium falciparum* sporozoites (CPS). In **Chapter 6** we assessed the life-cycle stage-specificity of CPS-induced protection and show that CPS immunization does not protect against an intravenously administered blood-stage challenge infection. The absence of protection against *P. falciparum*-infected erythrocytes indicates that CPS immunization only provides functional immunity against parasite liver-stages, emphasizing the need for a 100% effective CPS liver-stage immunization if this model is to be translated into an actual vaccine. In **Chapters 7 and 8** we determined the safety and tolerability of intradermal administration of cryopreserved *P. falciparum* sporozoites to volunteers taking chloroquine chemoprophylaxis, and its protective efficacy against infectious mosquito bites. We show that the current intradermal immunization regimen is safe and well tolerated, although it did not confer protection against infectious mosquito bites. This is in contrast to previous CPS studies in which volunteers were protected against infectious mosquito bites after CPS immunization. We believe that differences in efficiency of administration and infectivity of sporozoites between the regular CPS immunization and the currently used intradermal injections are likely responsible for the observed differences.

Each of the treatment and vaccination strategies described in this thesis has the potential to make a significant contribution to malaria elimination (and eventually eradication). To

achieve malaria elimination, i) access to highly sensitive and accurate diagnosis is essential in order to find all malaria-infected individuals, and diagnostics should be suitable for mass screening, ii) parasite positive individuals should be treated and onwards transmission should be prevented, and iii) gains should be sustained and herd immunity should be induced in low endemic and elimination areas. Strategies described in this thesis, could be applied into this three-step approach: i) RDTs could increasingly be used to identify geographical clusters of malaria infections ii) that could then be targeted with IVM and first line antimalarial drugs in a mass drug administration approach to prevent the spread of (resistant) parasites to surrounding populations, and eventually iii) a whole-sporozoite vaccine (once developed) could protect the population against future malaria outbreaks.

The future of malaria control and eradication efforts will be highly dependent on how well the scientific and public health communities, policy makers and local governments can work together to extend the effective life span of our existing tools while discerning new interventions that interrupt the complex life cycle of *Plasmodium* parasites.

Samenvatting

Het terugdringen van malariatransmissie wereldwijd is één van de grootste uitdagingen voor de internationale gezondheidszorg. Hoewel er aanzienlijke vooruitgang is geboekt, zullen de momenteel beschikbare kennis, middelen en strategieën in de meeste endemische gebieden waarschijnlijk niet leiden tot eliminatie van malaria. Dit is deels te wijten aan de toenemende resistentie van *Anopheles*-muggen tegen insecticiden en de verminderde gevoeligheid van de *Plasmodium*-parasiet voor diverse antimalariamiddelen. Om de verspreiding van resistente *Plasmodium*-parasieten tegen te gaan, zullen krachtigere middelen nodig zijn. Ook hebben de ontwikkeling en distributie van een werkzaam vaccin dat invloed heeft op malaria-incidentie, -ziektelast, en -mortaliteit hoge prioriteit.

In dit proefschrift evalueer ik momenteel beschikbare en experimentele behandelings- en vaccinatiestrategieën die malariabeheersing, -eliminatie, en -eradicatie nu en in de toekomst zouden kunnen ondersteunen. Mijn proefschrift bestaat uit drie delen.

- A. Verbetering van de beschikbaarheid en nauwkeurigheid van diagnostiek met behulp van malaria-sneltesten, een momenteel toegepaste strategie
- B. Toevoeging van muggendodende middelen aan de bestaande malariabehandeling om transmissie via de mug te voorkomen, een strategie voor de nabije toekomst
- C. Bescherming tegen malaria door inoculatie met sporozoïeten, een strategie voor de toekomst

In **Deel A** laten wij zien dat malaria-sneltesten (rapid diagnostic tests, RDTs) kunnen bijdragen aan een rationele behandeling van malaria. In gebieden waar men uitsluitend behandelde op basis van klinische symptomen maakt een RDT het mogelijk om de diagnose malaria te stellen door detectie van specifieke *Plasmodium*-antigenen. In **Hoofdstuk 2** zagen wij een direct effect van een beleidswijziging van de Tanzaniaanse overheid, waarbij er RDTs werden verstrekt aan ziekenhuizen en malariabehandeling alleen mocht worden gestart na een positieve RDT-uitslag. Na deze beleidswijziging verminderde het aantal voorgeschreven antimalariamiddelen aanzienlijk in de twee regionale ziekenhuizen waar onze observatie plaatsvond. In **Hoofdstuk 3** tonen wij dat deze trend ook is waargenomen in andere studies, met name daar waar men voorheen uitsluitend behandelde op basis van klinische symptomen. Invoering van RDTs blijkt echter minder effect te hebben indien microscopische diagnose van malaria reeds plaatsvond. Ook belichten wij het *T3: Test, Treat, Track* initiatief van de Wereldgezondheidsorganisatie dat zich richt op het gebruik van RDTs. Wij zijn van mening dat adequate en permanente bevoorrading met RDTs alsmede een gedegen opleiding voor al het gezondheidszorgpersoneel de grootste uitdagingen vormen voor RDT-implementatie in endemische gebieden.

In **Deel B** tonen wij een nieuwe manier om de transmissie van parasieten tussen mens en mug na standaard malariabehandeling te verhinderen. Deze transmissie is het gevolg van de aanwezigheid van gametocyten (seksuele stadia van de parasiet) in humaan bloed. Gametocyten zijn relatief ongevoelig voor eerstelijns antimalariamiddelen die daarom ondersteund moeten worden met middelen die transmissie voorkomen. De zoektocht naar deze transmissieblokkerende middelen heeft zich tot op heden gericht op middelen die gametocyten doden. Wij hebben aangetoond dat ivermectine (IVM, een geneesmiddel dat op grote schaal wordt gebruikt voor behandeling van rivierblindheid) malariatransmissie op onconventionele wijze kan verhinderen: IVM doodt de vector van de malariaparasiet, de *Anopheles* mug, in plaats van de gametocyt. Uit dierstudies beschreven in **Hoofdstuk 4** blijkt dat IVM een kortdurend maar evident muggendodend effect heeft. In **Hoofdstuk 5** observeerden wij in een dubbelblinde, placebo-gecontroleerde trial in Burkina Faso eenzelfde effect in de mens. In deze trial laten wij zien dat de combinatie van IVM met de huidige eerstelijns antimalariamiddelen veilig is en de overleving van *Anopheles* muggen significant verkleint. Het muggendodende effect van één en twee doses IVM resulteerde in dalingen van respectievelijk 27% en 35% van het malariatransmissiepotentieel tijdens de eerste week na start van behandeling. Wij zijn van mening dat deze combinatietherapie in de nabije toekomst kan worden gebruikt voor de bestrijding van malaria. Er dienen echter aanvullende studies te worden uitgevoerd om de precieze rol van IVM in elk type malaria-beheersingsstrategie te bepalen. Het definitieve bewijs zal moeten worden geleverd door cluster-gerandomiseerde community trials waarin een eenmalige of herhaalde dosis IVM bij voorkeur met een eerstelijns antimalariamiddel aan de hele populatie wordt gegeven.

In de **Deel C** beschrijven wij twee gecontroleerde humane malaria-infectiestudies waarin gezonde proefpersonen terwijl zij chloroquine innemen, worden blootgesteld aan *Plasmodium falciparum* sporozoïeten via muggenbeten (de conventionele CPS-immunisatie) of via injecties (een alternatieve wijze van CPS-immunisatie). Op deze manier proberen wij hen immuun te maken voor een nieuwe malaria-infectie. In **Hoofdstuk 6** laten wij zien dat CPS-immunisatie wél bescherming biedt tegen met sporozoïeten geïnfecteerde muggenbeten, maar niet tegen intraveneus toegediende bloedstadiumparasieten. Het ontbreken van bescherming tegen deze bloedstadiumparasieten duidt erop dat CPS-immunisatie alleen bescherming biedt tegen de leverstadia van de parasiet. In **Hoofdstuk 7 en 8** hebben wij bekeken of proefpersonen die intradermaal sporozoïeten krijgen toegediend en chloroquine innemen, immuun worden voor infectieuze muggenbeten. We concluderen dat het huidige intradermale immunisatiebeleid veilig is en goed verdragen wordt. Het biedt echter geen bescherming tegen infectieuze muggenbeten, in tegenstelling tot eerdere CPS-immunisatiestudies waarin proefpersonen immuun werden. Wij denken dat het verschil tussen de conventionele CPS-immunisatie en de intradermale injecties te verklaren is door een andere toedieningswijze en de mate waarin sporozoïeten infectieus zijn.

Elk van de behandelings- en vaccinatiestrategieën beschreven in dit proefschrift heeft de potentie om een belangrijke bijdrage te leveren aan malaria-eliminatie (en uiteindelijk eradicatie). Om malaria-eliminatie te bereiken, i) is toegang tot zeer gevoelige en nauwkeurige diagnostiek essentieel om alle malariageïnfecteerde individuen op te sporen en moet diagnostiek geschikt zijn voor massale screening, ii) dienen malariageïnfecteerde individuen te worden behandeld en moet verdere transmissie worden voorkomen, en iii) dient gemaakte progressie te worden gewaarborgd en immuniteit op populatieniveau te worden opgewekt in laagendemische gebieden en eliminatiegebieden. De strategieën beschreven in dit proefschrift kunnen worden toegepast in deze drietraps-aanpak: i) RDTs kunnen worden gebruikt om geografische clusters van malaria-infecties op te sporen ii) waar vervolgens zoveel mogelijk individuen met IVM en eerstelijns antimalariamiddelen worden behandeld om verspreiding van (resistente) parasieten naar omliggende populaties te voorkomen en iii) uiteindelijk zou een vaccin gebaseerd op sporozoïeten (eenmaal ontwikkeld) de populatie kunnen beschermen tegen toekomstig uitbraken van malaria.

De toekomst van malariabeheersings- en eradicatie-inspanningen zal sterk afhangen van de mate waarin wetenschap, volksgezondheidsorganisaties, beleidsmakers en lokale overheden samenwerken om de levensduur van bestaande beheersingsmaatregelen te verlengen en nieuwe interventies te ontwikkelen.

APPENDICES

LIST OF PUBLICATIONS

DANKWOORD

CURRICULUM VITAE

Appendix I - List of publications

Ouédraogo AL*, **Bastiaens GJ***, Tiono AB, Guelbéogo WM, Kobylinski KC, Ouédraogo A, Barry A, Bougouma EC, Nebie I, Ouattara MS, Lanke KH, Fleckenstein L, Sauerwein RW, Slater HC, Churcher TS, Sirima SB, Drakeley C, Bousema T. Efficacy and safety of the mosquitocidal drug ivermectin to prevent malaria transmission after treatment: a double-blind, randomized clinical trial. *Clin Infect Dis*. 2014 Nov 19

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* These authors contributed equally.

Appendix II - Dankwoord

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Lieve **papa** en **mama**. Jullie hebben alle ups en downs van mijn leven wel zo'n beetje meegemaakt en mij altijd onvoorwaardelijk gesteund. Ik kom nog altijd graag naar het zuiden. Niet alleen voor de vlai, maar ook om lekker bij te kletsen met een kopje koffie in de zon. En aanstaande zomer kunnen jullie lekker genieten van jullie kleinkind spelend op het gras!

Lieve schat, wij kennen elkaar nu al meer dan 9 jaar. Normale mensen zouden zich misschien al lang kapot vervelen, maar wij blijven maar praten! Jij hebt mijn promotie ups en downs van dichtbij meegemaakt, steunde me op de moeilijke momenten, hielp me te relativieren en vierde feest met mij op de mooie momenten. Afgelopen december hebben wij er een supermooi cadeautje bij gekregen toen onze dochter **Liselot** werd geboren. Ik heb nu al zin om met jullie door Burgers' Zoo te banjeren. Ik hou van jullie, kus!

Appendix III - Curriculum vitae

Guido Joseph Henri Bastiaens was born on the 11th of February 1984 in Heerlen, The Netherlands. In 2002 he passed his Gymnasium exam at the Bernardinuscollege in Heerlen and started studying Biology at the Radboud University Nijmegen. After receiving his propaedeutic diploma, Guido switched to study Medicine in 2003.

In 2005 he worked as a medical student in various health facilities in Tamale, Ghana (Bruham Clinic, Free and Fair Pharmacy and SDA Hospital) where he also conducted a public health-oriented research (prof. dr. E. Roscam Abbing). In 2007 he conducted a research on maxillofacial trauma in the department of Maxillofacial Surgery of the Muhimbili Medical Centre in Dar es Salaam, Tanzania (prof. dr. M.A. Merckx and dr. E.N. Simon). In this period he was (vice-)chairperson of the student branch of the Nijmegen Institute for International Health and he followed the Interdisciplinary Honours Programme at the Radboud University Nijmegen from which he graduated in 2008. In 2009 Guido followed an internship in Tropical Medicine in northern Tanzania (dr. M. Keuter), where he also conducted his first malaria research in three district hospitals (dr. T. Bousema, dr. S.A. Shekalaghe). Upon returning to the Netherlands, he obtained his medical degree in 2010 and started working as a resident in the department of General Surgery of the Slingeland Hospital as part of the training in Tropical Medicine.

Guido started his PhD in the department of Medical Microbiology of the Radboud University Medical Center in 2011, of which the research is described in this thesis (prof. dr. R.W. Sauerwein and dr. T. Bousema). During this period he conducted clinical malaria trials in the Netherlands and Burkina Faso, and fieldwork in Kenya. Since 2011 he is also a member of Uniting Streams, the working group for young field researchers of the Dutch Society of Tropical Medicine.

As of October 2014, Guido is working as a resident in Medical Microbiology at the Radboud University Medical Center.

Guido is married to Else Bastiaens-Eleveld and they have one daughter, Liselot, born on the 13th of December 2014.

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